ASSIMILATE PARTITIONING AND ENZYMES OF ORGANIC ACID METABOLISM IN FRUIT OF CALAMONDIN AND LOW-ACID GRAPEFRUIT

BY

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Organic acid formation in citrus fruit was characterized with respect to light and dark $^{14}CO_2$ fixation and enzymes of organic acid metabolism to quantify the sources of organic acids and identify possible mechanisms controlling acid accumulation in citrus fruit. Results were compared throughout development of grapefruit from normal- and low-acid clones and also in fruit of calamondin trees treated to reduce fruit acidity.

The ^{14}C-assimilates translocated from leaves to fruit were deposited primarily in juice tissues regardless of whether $^{14}CO_2$ fixation had occurred in light or dark. The majority of ^{14}C-photosynthates originating in fruit remained in peel tissues. During most of development, assimilates from dark $^{14}CO_2$ fixation by fruit were localized primarily in juice tissues and little subsequent redistribution occurred. Leaf photosynthates were the primary C source

for fruit growth, while the fruit photosynthates mainly contributed to growth of peel (flavedo and albedo).

Sources of organic acids were the same as those of total C only in very young fruit. Leaf photosynthates were the primary source of organic acids throughout early fruit development. However, with fruit expansion, assimilates from dark CO_2 fixation by fruit became one of the major, and possibly the predominant, source of organic acids. This was evident despite the minimal contribution to fruit C by dark CO_2 fixation.

Assimilate partitioning into organic acids was altered in leaves, but not fruits of arsenate-treated calamondin trees, and a greater portion of leaf ^{14}C-photosynthates was recovered in fruits. The contribution of fruit photosynthesis to growth of calamondin juice tissues appears to be greater than that of grapefruit (23% vs. less than 4%) and may be related to differences in peel thickness.

Examination of enzyme activities indicated that dark CO_2 fixation via the combined reactions of phosphoenolpyruvate carboxylase (PEPc) and malate dehydrogenase (MDH) was an important source of organic acid synthesis, but overall control of organic acid accumulation apparently occurred at steps other than the PEPc-MDH pathway. The greater activity of citrate synthase and lesser activity of aconitase in tissues with high acid levels, as well as the reverse, indicated that these two reactions together may be important in the control of synthesis and degradation of citrate in citrus fruit.

CHAPTER I
INTRODUCTION

Organic acids play a central role in plant metabolism. Citric acid and malic acid, which are the main organic acids in citrus fruit, are not simply the intermediates of respiration. Malic acid is also involved in photosynthesis (Edwards and Huber, 1981; Ting, 1985), pH balance (Davies, 1979; Bown, 1985), and nitrogen assimilation (Raven and Smith, 1976; Robinson, 1983; Lance and Rustin, 1984). Many fruits are particularly rich in organic acids which are usually dissolved in the cell water, either as free acids or combined as salts, esters or glycosides (Ulrich, 1970).

Acid content is an important quality factor in fruit and can be affected by genetic, developmental and environmental factors. The ratio of soluble solids to total acids is also a major criterion of maturity for citrus and certain other fruits (Ulrich, 1970; Sinclair, 1984). Citric and malic acids predominate in citrus fruit, but oxalic, malonic, succinic and other minor acids can also be found (Kefford and Chandler, 1970; Monselise and Galily, 1979). Citric acid is predominant in fruit of sour citrus species while malic acid is more prevalent in those with sweeter fruit (Kefford and Chandler, 1970). Organic acids accumulate throughout the development of citrus fruit (Ting and Vines, 1966; Erickson, 1968; Kefford and Chandler, 1970; Sinclair, 1984), but the rate and timing of accumulation vary with different species.

Three hypotheses have been proposed to account for the origin of organic acid accumulated in fruit:

1. Organic acids are formed within fruit via metabolism of photosynthates transported from leaves (Varma and Ramakrishnan, 1956; Ramakrishnan, 1971).

2. Organic acids might be transported directly from leaves or roots as in grapevine (Ulrich, 1970).

3. Organic acids are products of CO_2 fixation by fruit, either directly from phosphoenolpyruvate(PEP) carboxylase activity in juice tissues (Huffaker and Wallace, 1959), and/or metabolism of carbohydrates produced during C_3 photosynthesis in fruit.

Recent research tends to favor the first hypothesis. Evidence indicates that some organic acids are produced from photosynthates imported into fruit and accumulation can presumably occur if there is an block in metabolism (Buslig, 1971; Ramakrishnan, 1971; Kubota and Akao, 1973a). Overproduction of organic acids and/or slow utilization would need to be followed by uptake and storage inside vacuoles. Still, no quantitative data describe the extent to which this occurs or confirm a specific source of fruit acids.

Less data exist on transport of organic acids from leaves to fruits. Organic acid levels are quantitatively a minor constituent of sieve-tube sap and initially seem insufficient to explain the large accumulation in fruit (Ziegler, 1975). However, organic acids comprise only 1.2% to 1.8% of the fresh weight in grapefruit and 4.4% to 6.4% in lemon juice (Sinclair, 1984). They may make up 2.0 to 3.2 mg/ml of the phloem sap (Hall and Baker, 1972). A major portion of grape acidity has been found to originate in this way (as reviewed by

Ulrich, 1970).

Fixation of CO_2 in fruit can also give rise to organic acids, especially if initial carboxylation occurs via PEP carboxylase (Huffaker and Wallace, 1959; Bean and Todd, 1960). This enzyme is known to be active in citrus fruit (Huffaker and Wallace, 1959; Bogin and Wallace, 1966a). Huffaker and Wallace (1959) first suggested that dark CO_2 fixation could be related to acid accumulation in citrus fruit and that PEP carboxylase was a key enzyme in CO_2 fixation. Bean and Todd (1960) further identified labeled organic acids in young lemon fruit after dark $^{14}CO_2$ fixation. However, the relationship between amounts of CO_2 fixation and fruit acidity differed depending on fruit tissues examined and methods used (Clark and Wallace, 1963; Bogin and Wallace, 1966a). Still, overall photosynthetic rates (via RuBP carboxylase + PEP carboxylase) were greatest in young citrus fruit during the time when much organic acid accumulation occurred (Kidd and West, 1947; Bean and Todd, 1960; Todd et al., 1961; Kriedemann, 1968a; Pandey and Farmahan, 1977; Moreshet and Green, 1980; Chauhan and Pandey, 1984). Total fruit photosynthesis is believed to contribute little to development of the organ (Kidd and West, 1947; Todd et al., 1961, Schaedle, 1975; Chauhan and Pandey, 1984), but the amount of organic acids derived either directly or indirectly from this process within fruit is unknown.

Photosynthate partitioning in whole plants has been extensively studied (as reviewed by Wardlaw, 1968; Wareing and Patrick, 1975; Geiger and Giaquinta, 1982; Daie, 1985; Delrot and Bonnemain, 1985), but little attention has been directed toward the identity, quality, or distribution of assimilates produced in fleshy, acid-accumulating

fruit either from light or dark CO_2 fixation (Bollard, 1970). The relative amount of fruit photosynthates partitioned into organic acids remains unclear.

Two additional approaches to determining the sources of fruit acid production are made possible by the existence of genetic and chemical mechanisms which alter fruit acidity. One grapefruit mutant with only 2/3 the normal acid levels was developed by bud irradiation of 'Foster' pink grapefruit (Hearn, 1986). Changes in accumulation and metabolism of organic acid could thus be compared between this low-acid mutant and a normal-acid counterpart which differ in acid levels. Arsenate spray is also known to substantially decrease acidity in grapefruit and is widely used in Florida (Wilson, 1983). Differences in metabolism and CO_2 fixation by normal vs. low acid grapefruit clones and between arsenated and non-arsenated calamondin trees may provide information valuable to understanding the control of acid accumulation in citrus fruit.

The objectives of this research are to quantify the extent to which fruit organic acids are produced by metabolism of leaf photosynthates transported to fruit, direct translocation of organic acids from leaves to fruit, and light and dark CO_2 assimilation in fruit during their development. The additional objectives are to compare these processes as well as activities of acid metabolizing enzymes in normal- and low-acid grapefruit mutants, and characterize related effects of arsenate on acid accumulation in citrus fruit.

CHAPTER II
LITERATURE REVIEW

Assimilate Partitioning in Citrus

Assimilate translocation out of leaves has been extensively studied in many plant species and food crops. The proximity of a given source leaf to a sink has long been known to affect the extent of photosynthate export and its subsequent distribution in the plant (Wardlaw, 1968; Wareing and Patrick, 1975). A greater proportion of the assimilates from leaves near roots will move below ground, while photosynthates from upper leaves will be translocated preferentially to the shoot apex. Assimilates can move either or both directions from leaves at an intermediate position (Wardlaw, 1968). A similar pattern of photosynthate distribution was also found in citrus (Powell, 1968; Kriedemann, 1969a, 1969b, 1970). Assimilates exported from leaves on the same growth flush with fruit move almost exclusively into these fruit (Powell, 1968). Leaves farther from fruit, however, export assimilates in both acropetal and basipetal directions, while ^{14}C-photosynthates derived from leaves near the soil line show extensive basipetal movement (Powell, 1968; Kriedemann, 1969a, 1969b; Powell and Krezdorn, 1977; Brown, 1974).

The proportion of ^{14}C-assimilates received by navel orange fruit from adjacent leaves also changes seasonally. Variations are apparently affected by both the position of leaves and competition between newly developing leaves and fruits (Brown, 1974). Initial

flushes are the strongest sinks, followed by the developing fruits. Developing leaves represent the most powerful sinks and do not become contributing organs until fully mature (Kriedemann, 1969a, 1969b; Kadoya, 1974). This pattern of assimilate partitioning is different from that in other fruit crops in which leaves typically export assimilates when only partially expanded (Bollard, 1970). The application of gibberellic acid and cytokinin can increase the import of assimilates or replace the fruit as a sink when fruit is removed (Powell, 1968; Kriedeman, 1968a; Powell and Krezdorn, 1977; Brown, 1974; Kadoya, 1974). Photosynthate partitioning within leaves and allocation in the whole plant can affect the growth of fruits, which in turn can influence not only the direction and rate of translocation but also leaf photosynthetic rate (Kadoya, 1974; Lenz, 1979).

Transport of ^{14}C-assimilates into citrus fruit was initially believed to occur by way of the vascular tissue and hence movement into juice sacs would be via the vascular bundles on the surface of the segment epidermis (Kriedemann, 1969a). Anatomical and radiolabeling studies of grapefruit showed that the majority of photosynthates translocated into juice sacs moved via dorsal vascular bundles (Koch, 1984b). The narrow band of distribution for photosynthates entering citrus fruit from an individual leaf demonstrates a possible translocational basis for the unequal distribution of soluble solids in citrus fruit (Koch, 1984a). Sucrose is apparently the primary transport sugar in citrus phloem (Kriedemann, 1969a; Koch, 1984b).

Export of ^{14}C-assimilates from citrus leaves can be rapid, but also may occur for an extended period. Measurable import of

^{14}C-assimilates into fruits was detected 20 min after exposure of adjacent leaves to $^{14}CO_2$ (Kriedemann, 1969b, 1970). Still, mature leaves were able to export labeled photosynthate for at least 40 days after $^{14}CO_2$ administration (Kriedemann, 1969b, 1970). The majority of assimilates translocated from leaves to adjacent grapefruit are transferred within 24 to 30 h, but export appears to occur more rapidly during greatest rates of fruit expansion (Koch, unpublished data). Fruit photosynthesis also occurs in citrus but declines toward maturity (Bean and Todd, 1960; Todd et al., 1961; Ramakrishnan, 1971; Moreshet and Green, 1980). Assimilation of CO_2 per unit area in fruit is 25-50% of that by leaves in 'Valencia' orange (Moreshet and Green, 1980). The uptake was dependent on fruit size, photosynthetically active radiation, chlorophyll content, and on different diffusive conductances of the fruit epidermis (Moreshet and Green, 1980). Fruit photosynthesis in oranges and lemons contributes little toward fruit development (Todd et al., 1961; Moreshet and Green, 1980). Similar results were also found in other fruit crops such as apple, avocado and mango (Kidd and West, 1947; Pandey and Farmahan, 1977; Chauhan and Pandey, 1984). Labeled organic acids were detected after $^{14}CO_2$ fixation in young citrus fruit in light, but the amount was relatively low compared to the labeled sucrose content (Bean and Todd, 1960). The extent to which fruit photosynthate is partitioned into organic acids throughout development remains unclear.

Organic acid production during CO_2 assimilation in the dark is well documented in fleshy tissues of desert plants (Edwards and Huber, 1980; Ting, 1985). Uptake of CO_2 in darkness is essential to photosynthetic processes in succulent plants which utilize

Crassulacean Acid Metabolism (CAM) to conserve water during carbon assimilation. Dark CO_2 fixation is also known to be an anaplerotic carboxylation reaction in other tissues and is related to pH balance in cells (Davies, 1973; Bidwell, 1983; Bown, 1985). Phosphoenolpyruvate carboxylase (PEPc) is the enzyme most commonly responsible for CO_2 fixation in the dark (Huffaker and Wallace, 1959; Bown, 1985; Ting, 1985). Data of Bean and Todd (1960) indicated that the ^{14}C-assimilates formed by citrus fruit in the dark must be produced within each tissue rather than by extensive translocation from one to another. More labeled-assimilates were found in pulp of young intact lemon fruit than other fruit tissues after dark $^{14}CO_2$ fixation. Assimilates were partitioned mainly into organic and amino acids.

Organic Acids in Citrus Fruits

Organic acids are involved in almost all aspects of plant metabolism. These have been extensively reviewed in relation to specific aspects of the metabolism in which they are involved (Thimann and Bonner, 1950; Bartholomew and Sinclair 1951; Kefford 1959; Sinclair, 1961; Beevers et al., 1966; Kefford and Chandler, 1970; Ramakrishnan 1971; Ting and Attaway, 1971; Sinclair, 1972; Vandercook, 1977; Sinclair, 1984). Citric and malic acids are respiratory intermediates. Malate, which is also produced via CO_2 fixation by PEP carboxylase, plays an important role in photosynthesis (Edwards and Huber, 1981; Ting, 1985), pH balance (Davies, 1973; Bown, 1985), and nitrogen assimilation (Raven and Smith, 1976; Robinson, 1983; Lance and Rustin, 1984). It can also transport reducing equivalents across

membranes (Lance and Rustin, 1984).

Both citric and malic acids are the major organic acids in citrus fruit, but the type and levels of acids can vary greatly among different geographic locations and tissues (Sinclair and Eny, 1947; Ting, 1969; Monselise and Galily 1979). Citrate is the primary acid in grapefruit juice while malate is the dominant acid in peel (both flavedo and albedo) (Monselise and Galily, 1979). Acidity is also unevenly distributed in the juice tissues, and higher levels are found toward the core region of grapefruit and oranges (Ting, 1969).

Fruit acidity is affected by both genetic and horticultural factors. Acid content can be as high as 6% (expressed as citric acid/fresh weight) in lemon fruit or less than 0.1% in acidless orange or pummelo (Cameron and Soost, 1977; Sinclair, 1984). Fruits produced on trees with a trifoliate orange rootstock are generally higher in acid content than those from trees on rough lemon rootstock (Sinclair, 1984). Citrus fruit grown at lower temperatures are higher in acidity than those produced in warmer climates or during warmer years (Reuther, 1973). Application of higher levels of nitrogen and potassium fertilizers also increases fruit acidity (Vandercook, 1977). In addition, irrigation significantly decreases the acidity in grapefruit and oranges compared with unirrigated groves (Koo and McCornack, 1965; Gilfillan et al., 1976). Arsenate spray about 1 month after blooming has long been known to decrease acid content in citrus fruit (Deszyck and Ting, 1958; Wilson, 1983; Rice et al., 1985). On the contrary, boron or copper sprays will increase the acidity of citrus fruit and often have an effect antagonistic to that of arsenate (Smith, 1973).

Organic acids are accumulated throughout fruit development (Ting and Vines, 1966; Kefford and Chandler, 1970; Sinclair, 1984), but timing and rate of accumulation vary with different citrus species. Acids accumulate more rapidly in young, developing fruit (Erickson, 1968). Total acidity increases during fruit growth; however, the concentration remains stable or decreases toward maturity (Ting and Vines, 1966; Erickson, 1968).

Organic Acid Biosynthesis in Citrus Fruits

Reciprocal grafts between sweet and sour lemons have suggested that the mechanism affecting organic acid accumulation might be localized primarily, but not entirely in fruit. Sweet lemon fruits grafted to sour lemon plants remained low in citric acid (Erickson, 1957), but acidity of these fruit was higher than when the same sweet lemons were grafted to sweet lemon plants. These data indicate that the leaves and fruit can both affect the acidity of citrus juice (Erickson, 1957).

Lack of acid accumulation in peels of citrus fruit led Sinclair and Eny (1947) to suggest that organic acids were probably synthesized in the juice sacs from carbohydrates. Ramakrishnan (1971) later proposed that carbohydrates were utilized by a combined operation of both the glycolytic pathway and TCA cycle, and the citric acid would therefore be expected to accumulate due to an absence of aconitase activity. Labeled citrate was found shortly after radioactive carbohydrates were supplied to fruits (Ulrich, 1970). Citric acid and to a lesser extent malic acid were the main intermediates accumulated when the TCA cycle was blocked by different metabolic inhibitors in

satsuma mandarin fruit (Kubota and Akao 1973a). Activity of the TCA cycle was observed in several citrus fruits after addition of labeled citrate, succinate and pyruvate (Bogin and Erickson, 1965; Kubota and Akao, 1973a; Akao and Kubota, 1978b). A comparison between the amount of labeled malate and citrate formed after adding labeled pyruvate indicated that more of the pyruvate entering the TCA cycle had arisen indirectly from CO_2 fixation than that which entered via the citrate synthase reaction (Bogin and Wallace, 1966a, 1967).

Citrate synthase is a major regulatory enzyme in the TCA cycle and Bruemmer et al. (1977) have suggested that its activity could be responsible for controlling citric acid production in citrus fruit. Buslig (1971) indicated that an asynchrony between activities of citrate synthase and the degradative enzymes, aconitase and isocitrate dehydrogenase, might explain the accumulation of citrate in citrus fruit. Bogin and Wallace (1967) proposed that higher citric acid levels in sour lemon were due to reduction of aconitase activity by citramalate, an inhibitory acid formed in citrus from pyruvate. The amount of pyruvate available for citramalate formation would presumably decrease under circumstances favoring higher CO_2 fixation by PEP carboxylase or more rapid conversion of pyruvate to alanine (Bogin and Wallace, 1967). No further study has been reported on this hypothesis.

Another contributing factor could be that, as in a series of experiments with grape (Ruffner, 1982; Ruffner et al., 1983), the reduction of acid formation during ripening was found to be due to the inhibition of glycolytic carbon flow. This in turn resulted in accumulation of imported sugars. At the same time, the ripening fruit

could transform acids into sugars by reactions involving a reversal of the glycolytic pathway (Ruffner et al., 1975).

Although organic acids are minor components in phloem sap, most organic acids such as citric, malic, oxaloacetic and tartaric acids found in fruit have been detected in exudates from phloem. The type of organic acids varies with plant species (Ziegler 1975). Malate in phloem of Ricinus plants is believed to be associated with the maintenance of ionic balance (Hall and Baker, 1972). Also, the direct translocation of organic acids from leaves has been demonstrated in grape (as reviewed by Ulrich, 1970). The contribution of organic acids translocated from leaves or roots to acid accumulation in citrus fruit is unknown.

The role of dark CO_2 fixation in organic acid biosynthesis has been debated since Huffaker and Wallace (1959) first suggested that it might be associated with the accumulation of acid in citrus fruit. Subsequent studies identified labeled organic acids in young lemon fruit after dark $^{14}CO_2$ fixation (Bean and Todd, 1960). However, the amount of dark CO_2 fixation did not correlate with the acidity of fruit tissues when different species, fruit parts, and methods were used (Clark and Wallace, 1963; Bogin and Wallace, 1966a). Clark and Wallace (1963) found sweet lime, which was lowest in acid content, had the highest CO_2 fixation rate in juice vesicle homogenates. Bogin and Wallace (1966a) found that CO2 fixation was greater in sweet lemon extracts when crude PEP carboxylase, malic enzyme and isocitrate dehydrogenase were used, whereas maximal rates were observed in sour lemon when entire peeled fruit was used. In Citrus natsudaidai, either in light or dark, the amount of CO_2 fixation in sweet fruit juice

vesicles was greater than in sour fruit (Akao and Tsukahara, 1979). In contrast, CO_2 fixation in juice vesicles was lower in arsenate treated fruit (Akao and Kubota, 1978a). Despite differing results, it was agreed that dark CO_2 fixation was greater during the period of rapid organic acid accumulation in fruit and declined toward maturity, as in grape berry (Kriedemann, 1968a).

Further research on different citrus fruit showed that organic acids (mainly citric and malic) were labeled after dark CO_2 fixation (Bean and Todd, 1960; Young and Biale, 1968; Akao and Kubota, 1978b; Akao and Tsukahara, 1980a, b). Isolated juice vesicles were found to accumulate more ^{14}C-assimilates than flavedo or albedo during dark $^{14}CO_2$ fixation. Also, more labeled citrate and malate were found in juice vesicles than in albedo and flavedo (Bean and Todd, 1960). Products of $^{14}CO_2$ assimilation in the dark seemed to be formed within each tissue rather than by extensive translocation from one tissue to another (Bean and Todd, 1960).

Arsenate Treatment in Citrus

Asenate sprays have been applied to Florida grapefruit trees for many years to decrease total acidity in juice vesicles at harvest (Miller et al., 1933; Deszyck and Ting, 1958, 1960; Wilson, 1983). The most effective application period is 1 to 6 weeks following bloom (Wilson, 1983). A similar effect has been identified in other citrus species such as orange, satsuma mandarin, and calamondin (Buslig, 1971; Akao and Kubota, 1978a; Wilson, 1983). The response to nonleaded arsenate was similar to those of lead arsenate indicating that arsenic was the major contributor to acidity reduction (Rice et al., 1985).

Most organic and inorganic arsenical compounds will cause similar effects (Wilson, 1983). In a whole tree, only sprayed portions are affected, suggesting the action of arsenate sprays is not broadly systemic. The response of actively growing leaves to arsenate spray was evident almost immediately in fruit and the effect persisted for about 18 months (Miller et al., 1933).

High levels of arsenic are toxic to both plants and animals, despite the role of arsenate as an essential nutrient for certain animal species (Wilson, 1983). Arsenate is known to uncouple phosphorylation and is used as a herbicide due to its influence on sulfhydryl-group enzymes (Vines and Oberbacher, 1965; Beevers et al., 1966; Knowles, 1982). Oxidation of pyruvate and α-ketoglutarate, both respiratory intermediates, is also influenced by arsenate (Beevers et al., 1966). Aerobic fermentation, as evidenced by greater CO_2 evolution and alcohol accumulation, was induced by arsenate treatment (Beevers et al., 1966). Vines and Oberbacher (1965) proposed that this uncoupling effect was the primary mechanism of acidity reduction in citrus fruit. Akao and Kubota (1978a) determined that the $^{14}CO_2$ fixation rate of juice vesicles from fruit sprayed with lead arsenate was lower than that of vesicles from unsprayed fruit. Similar results were also obtained in citrus juice tissues by Huffaker and Wallace (1959), but the activities of PEP carboxylase and malic enzyme in citrus leaves and fruit albedo were not affected or were only slightly increased. However, arsenic residue in juice sacs of sprayed fruit is usually negligible and normally not significantly different from that of unsprayed controls (Gilfillan et al., 1976; Rice et al., 1985). Lack of either lead or arsenate in juice sacs after postbloom sprays

led Rice et al. (1985) to conclude that the primary effects of arsenate spray might be elicited in the leaves. Gilfillan et al. (1976) compared the arsenic concentration in peel and pulp and found significantly higher arsenic levels in peel after treatment. The possible effects of higher arsenic in peel thus cannot be excluded.

Arsenate has long been known to increase the respiration of leaves in grapefruit. Decreased acidity was supposed to be associated with the increase of leaf respiration (Miller et al., 1933). Experiments on isolated pea chloroplasts indicated that arsenite affected light modulation and photosynthetic induction through its inhibition of light activation of enzymes in the reductive pentose phosphate (C_3) cycle (Marques and Anderson, 1986).

Boron has been reported to increase fruit acidity and show an antagonistic effect to arsenate. The overall antagonism was essentially the same when both elements were included in the same spray, applied in separate sprays, separated in a time interval, or when boron was applied to soil and arsenate to the foliage (Smith, 1973). However, Miller et al. (1933) indicated that soil application of arsenate was ineffectual in reduction of grapefruit acidity. The mechanism by which arsenate decreases acid levels in citrus fruit is unknown.

Enzymes Associated with Organic Acid Metabolism

Several enzymes are related to organic acid synthesis and degradation. They may be divided into two groups according to their functions and locations. Enzymes of malate synthesis are located in cytoplasm and mitochondria, while enzymes of citrate synthesis are

located mainly in the mitochondria.

Malate Synthesis from PEP

Three enzymes, PEP carboxylase (PEPc), malate dehydrogenase (MDH) and NAD-malic enzyme (NAD-ME) regulate the synthesis and oxidation of malate, which functions as a source of carbon for anaplerotic operation of the citric acid cycle (Lance and Rustin, 1984; Naik and Nicholas, 1986). Latzko and Kelly (1983) list eleven physiological functions dependent on the concentration of malate and the associated relationship between activity of PEPc and MDH.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) is almost ubiquitous in plants and plays a particularly important role in carbon fixation by C_4 and CAM plants (O'Leary, 1982). It is located in cytoplasm (Edwards and Huber, 1981) and catalyzes the reaction

$$PEP + HCO_3^- \text{ ------> } OAA + Pi$$

Normally, OAA is rapidly converted to malate in most plant systems. At least four different forms of PEP carboxylase exist in higher plants and are believed to be associated with the specific functions among plants. A PEP carboxylase from non-green root tissue was found to have a K_m and V_{max} considerably lower than that of the enzyme from leaves of CAM and C_4 plants (Ting and Osmond, 1973). Even in extracts from the same tissue, multiple forms of PEPc may exist that contribute to a diurnal variation in overall activity (O'Leary, 1982). The PEPc-MDH-ME enzymes also act as a cellular pH stat in plants (Davies, 1973). Concentration of malic acid in the cytoplasm depends on the balance between carboxylation (by PEPc) and decarboxylation (by ME). Phosphoenolpyruvate carboxylase has maximum activity near pH 8, while malic enzyme has maximum activity close to pH 6.5. When cellular pH

rises, PEPc is active and ME less so. At the same time, malate accumulates and releases two protons for each malate, thus balancing the increase of OH^- ions (Davies, 1973). Phosphoenolpyruvate activity was demonstrated in leaves, roots, fruit flavedo and juice vesicles of 'Valencia' orange (Huffaker and Wallace 1959). Huffaker and Wallace (1959) also proposed a pathway of citric acid synthesis in citrus fruit vesicles via a sequence of PEPc-MDH-citrate synthase. Later experiments in the same laboratory confirmed that dark CO_2 fixation rates were higher during the period of maximal acid accumulation. However, CO_2 fixation was also greater in low acid fruit, which is inconsistent with their hypothesis (Clark and Wallace, 1963; Bogin and Wallace, 1966a).

Malate dehydrogenase is found in chloroplasts, mitochondria, cytoplasm and microbodies, and catalyzes the reaction

OAA + NADH(NADPH) <------> malate + NAD(NADP)

The chloroplast enzyme is specific for NADP+, the others are specific for NAD+. Vine (1968) identified two forms of malate dehydrogenase (MDH) isolated separately from mitochondria and cytoplasm of grapefruit juice vesicles. The mitochondrial MDH was inhibited by elevated concentrations of oxaloacetic acid while the cytoplasmic MDH was insensitive. The enzymes of both sources were specific for L-malate and insensitive to D-malate.

Malic enzyme catalyzes the reaction

Malate + NAP(NADP) ------> pyruvate + CO_2 + NADH(NADPH)

Malic enzyme, like MDH, has many isozymes which are distributed in chloroplasts, mitochondria, and the cytoplasm, but the cytosolic and chloroplastic ME are specific for $NADP^+$, while the mitochondrial ME is

specific for NAD^+ (Edwards and Huber, 1981; Lance and Rustin, 1984). One of the differences between plant and animal respiration is that plants can form pyruvate and citrate from malate alone, through the combined action of MDH and malic enzyme (Palmer, 1976; Moller and Palmer, 1984). This mechanism allows the interconversion of all TCA cycle intermediates, thus the input of one intermediate can supplement the pool of any other intermediate (Moller and Palmer, 1984). Formation of pyruvate from PEP in plants is believed to occur to a greater extent via PEP carboxylase, MDH, and malic enzyme than through pyruvate kinase (Bogin and Wallace, 1966a; Lance and Rustin, 1984). Also, evidence indicates pyruvate is not effectively transported across the mitochondrial membrane in plants (Wiskish, 1980). Biosynthesis and oxidation of malate are regulated by NADH, OAA, pH, and adenylate balance of the cell. The control of MDH, ME and regulation of the malate/OAA concentration in the cell, as well as its relationship to cell energy charge and nitrogen assimilation have been reviewed by Wiskich (1980), Palmer (1984), Lance and Rustin (1984) and Naik and Nicholas (1986).

Citrate Synthesis and Subsequent Citrate Degradation in the TCA Cycle

Citrate synthase (EC 4.1.3.7, or citrate condensing enzyme, condensing enzyme, citragenase, OAA transacetase) is located in mitochondria and glyoxysomes and catalyzes the reaction

$$OAA + \text{acetyl-CoA} + H_2O \longrightarrow \text{citrate} + CoA$$

Aconitase (EC 4.2.1.3) catalyzes the interconversion of citrate, aconitate and isocitrate

$$\text{citrate} \longleftrightarrow \text{cis-aconitate} \longleftrightarrow \text{isocitrate}$$

The equilibrium constant favors the formation of citrate (Moller and

Palmer, 1984). Many reviews of citrate synthase and reactions associated with citrate have been presented (Srere, 1972; Srere, 1975; Weitzman and Danson, 1975; Wiskich, 1980; Moller and Palmer, 1984). Overwhelming data on this TCA cycle enzyme have been accumulated. Only the information related to organic acid accumulation in citrus fruit will be cited here.

Citrate synthase was found in leaves, peel and pulp of orange and lemon (Srere and Senkin 1966), grapefruit (Ting and Attaway, 1971) and satsuma mandarin (Hirai and Ueno 1977). Peel and pulp of green oranges were found to contain more of this enzyme than respective tissues from mature fruit. Activity of citrate synthase was also higher in peel than pulp (Srere and Senkin 1966).

An imbalance between activity of citrate synthase and subsequent TCA cycle enzymes, aconitase and isocitrate dehydrogenase, has been proposed to be responsible for the citrate accumulation in citrus fruit (Varma and Ramakrishnan, 1956; Ramakrishnan and Varma, 1959; Ramkrishnan, 1971; Buslig, 1971; Bruemmer et al., 1977). Far less is known about aconitase than citrate synthase. Palmityl CoA and ATP reportedly decrease the affinity of citrate synthase for oxaloacetate making the other enzyme more competive. The NADH is a negative effector of malate dehydrogenase and a potential regulator of citrate synthesis (Bogin and Wallace, 1966b; Bruemmer et al., 1977). Phosphoenolpyruvate carboxylase catalyzes an irreversible reaction; therefore it does not compete with citrate synthase and MDH for available oxaloacetate (Bruemmer et al., 1977). Citrate synthase activity increases, and aconitase and isocitrate dehydrogenase decrease, during the rise in acid content of lemons from 0.9% to 4.0 %

(Ramakrishnan and Varma, 1959).

Data of Vines and Metcalf (1967) indicated that variation in mitochondrial activity in grapefruit coincided with the rise and fall of total juice acidity, suggesting the possibility that changes in citric acid content may be correlated with the general phosphorylative ability of the fruit mitochondria. Hirai and Ueno (1977) followed the enzymatic changes of satsuma fruit during development and found that the ratio of phosphoenolpyruvate caboxylase and NAD-dependent isocitrate dehydrogenase was highest during most rapid acid accumulation. Isocitrate dehydrogenase may play an additional regulatory role in acid accumulation.

CHAPTER III
DISTRIBUTION OF ASSIMILATES PRODUCED DURING LIGHT AND DARK $^{14}CO_2$ FIXATION BY DEVELOPING GRAPEFRUIT AND ADJACENT SOURCE LEAVES

Introduction

The vast majority of assimilates are produced by leaves during photosynthesis in light, but CO_2 fixation occurs in fruits as well as leaves, and in both light and dark. Partitioning and translocation of photosynthates derived from source leaves of whole plants is relatively well-documented in a number of commercially important food species (Wardlaw, 1968; Wareing and Patrick, 1975). Less information is available for fleshy fruit crops, including citrus, but these fruit are known to be strong sinks for leaf photosynthates (Kriedemann, 1969a, 1969b; Kadoya, 1974). The presence of fruit can affect photosynthetic efficiency of citrus leaves and partitioning of photosynthates (Lenz, 1979). Assimilates are also preferentially translocated from citrus source leaves to aligned sections of fruit tissue (Koch and Avigne, 1984). In addition, photosynthates from leaves enter juice sacs very slowly, via a limited number of vascular bundles and extensive phloem-free areas of the transport path (Koch, 1984b; Koch et al., 1986). Seasonal alternations have been reported by Brown (1974) for leaf-derived assimilates arriving in navel oranges. Changes which occur in photosynthate translocation and distribution within these fruit have not been examined during their development,

however.

Photosynthesis also occurs in fruit of many crops. Studies of pea and soybean indicated that the pods could provide photosynthates to developing seeds by reassimilation of respiratory CO_2 (Quebedeaux and Chollet, 1975; Atkins et al., 1977). In a young green apple or citrus fruit, photosynthetic CO_2 fixation is of sufficient magnitude to conserve 20-80% of the CO_2 release by dark respiration depending on stage of development (Kidd and West, 1947; Bean et al., 1963; Shaedle, 1975).

Fruit photosynthesis was reported to decrease toward maturity in citrus (Bean and Todd, 1960; Todd et al., 1961; Ramakrishnan, 1971; Moreshet and Green, 1980). Contribution of fruit photosynthesis to orange and lemon growth is believed to be relatively small compared with that of assimilates translocated from leaves (Todd et al., 1961; Moreshet and Green, 1980). However, about 25% of the ^{14}C-photosynthates remain in satsuma mandarin juice tissues 5 days after fruit exposure to $^{14}CO_2$ in the light (Akao and Tsukahara, 1980a). Little information is available, however, on the partitioning, distribution, or redistribution of photosynthates produced by grapefruit or other fleshy fruit.

Dark CO_2 fixation, an anaplerotic reaction contributing to amino and organic acid formation (Bidwell, 1983), is involved in root growth (Splittstoesser, 1966), and flower bud development of 'Valencia' oranges (Vu et al., 1985). A similar relationship may exist for organic acid synthesis in citrus juice tissues. Minimal research has been directed toward analysis of assimilates arising from non-photosynthetic carboxylation reactions in fruit. However, greater

amounts of ^{14}C-assimilates were found in citrus juice sacs than surrounding tissues after each was exposed to $^{14}CO_2$ in the dark (Bean and Todd, 1960). Greater proportions of labeled organic acids (predominantly in citrate) and amino acids were also recovered in juice tissues after dark CO_2 fixation by fruit than in light. This process has long been considered one possible source of organic acid synthesis in citrus (Huffaker and Wallace, 1959; Bean and Todd, 1960; Young and Bial, 1968). The extent of dark CO_2 fixation in whole fruit has not been examined, nor have developmental changes in these reactions.

One purpose of the present work is to compare the distribution of labeled assimilates derived from $^{14}CO_2$ fixation in light and dark by fruit and adjacent source leaves throughout grapefruit development. The second is to assess the relative contributions of each assimilate source to a given stage of fruit growth.

Materials and Methods

Fruits and/or branches with source leaves adjacent the fruit were randomly selected from between 1 and 2 m above ground level on the exterior canopy of 4-year-old grapefruit trees ('Foster Pink' seedless clone # 1-26-39) on Savage sour orange or sour orange rootstocks in Lake Wales, FL (Hearn, 1986). No significant differences were evident in quality of fruit from trees on these two rootstocks (data not shown). Samples from a given tree were used for different analyses. Each tree represents one replication and four trees were used for each experiment. Fruit were harvested on May 7, June 25, July 27 and September 10, 1986 (at about 40-day intervals), after 2, 3.5, 5, and

6.5 months' growth, respectively. Pedicels and branch bases were immediately immersed in water. At each developmental stage, detached fruit or individual leaves adjacent to fruit were enclosed in a cuvette and exposed to 13.6 $\mu Ci\ l^{-1}$ $^{14}CO_2$ for 1 h in light followed by ambient air for 24h (12h:12h light:dark photoperiod). Levels of CO_2 were monitored during studies using an infrared gas analyzer (IRGA) (Model 865, Beckman Instruments Inc., Fullerton, CA) and a closed, flow-through system. Data were used to calculate mean specific activities of $^{14}CO_2$ during periods when respiratory CO_2 was increasing total amounts present. Temperature was maintained at 28°C and photosynthetic photon flux density at 800 to 1000 $\mu mol\ m^{-2}\ s^{-1}$. Light was provided by a Lucalox lamp (General Electric Co., Cleveland, OH). Similar experiments were conducted in 24 h of continuous darkness using cuvettes completely sealed with several layers of foil. Fruit were exposed to $^{14}CO_2$ within a few hours after pedicels or branches were removed from trees, and studies with source leaves were conducted the following morning.

Experiments were terminated by separating tissues into leaves and pedicels (if present), flavedo (outer, pigmented peel), albedo (inner, non-pigmented peel), and juice tissues. After some $^{14}CO_2$ treatments, fruit were halved longitudinally to compare the effect of leaf location or light source position on assimilate distribution in the fruit. Samples were frozen in liquid nitrogen, and extracted in 80% (v/v) ethanol. The ethanol-insoluble fraction was oven-dried for 4 days at 60°C. Radioactivity in each sample was quantified by liquid scintilation spectrometry (LKB, Gaithersburg, MD). The ethanol-soluble fraction was measured by pipetting 0.1 ml of sample into 15 ml

scintilation cocktail (Scinti Verse II, Fisher Scientific CO., Fair Lawn, NJ). The ethanol-insoluble fraction was measured by suspending a known portion of the sample in scintilation cocktail.

Results and Discussion

Fruit dry weight increased continuously during the developmental period examined, but did so most markedly during the 3rd and 4th months of growth (May to June) (Fig. 3-1A). Sink strength of fruit for photosynthates therefore was also greater at this time. Flavedo and albedo of peel accumulated the majority of their dry weight during this period and added only minute amounts at later stages. Increases in dry weight of juice tissues accounted for almost all change in total fruit dry weight during the second half of fruit growth and presumably also fruit demand for photosynthates.

Fresh weight gains during development (Fig. 3-1B) were proportional to those of dry weight, but more pronounced in juice tissues. Both fresh and dry weight followed trends similar to those observed in other citrus fruits (Bain, 1958; Sinclair,1984). The rapid increase in fresh and dry weight of fruit was typical of Stage II development observed in grapefruit (Lowell, 1986) and 'Valencia' oranges (Bain, 1958).

Sources of dry matter increase by grapefruit were compared throughout fruit development by examining ^{14}C-assimilate recovery in fruit 24 h after 1 h of light or dark $^{14}CO_2$ fixation by either leaves or fruit (Table 3-1). The total dpm recovered from all tissues after photosynthesis by a single leaf, as well as the percentage translocated to fruit, was by far the greatest at early stages of

Fig. 3-1. Developmental changes in dry weight (A) and fresh weight (B) of tissues in grapefruit ('Foster' pink seedless clone # 1-26-39). Vertical bars denote the SE of 4 replications, but are present only where larger than tissue/fruit symbols.

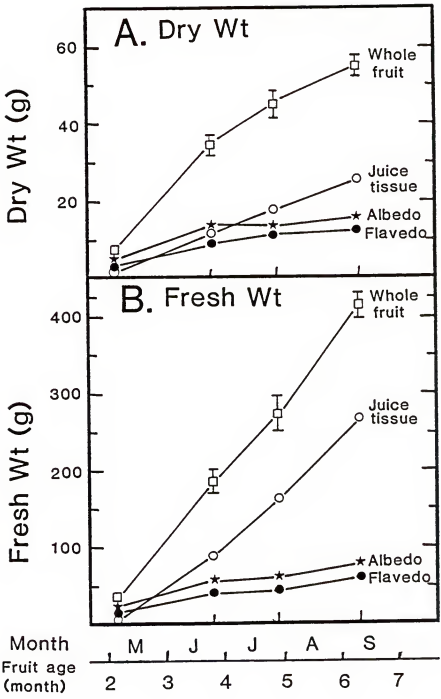

A. Dry Wt
Dry Wt (g)
60
40
20
Whole fruit
Juice tissue
Albedo
Flavedo
B. Fresh Wt
Fresh Wt (g)
400
300
200
100
Whole fruit
Juice tissue
Albedo
Flavedo
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

Table 3-1. The total C contribution and ^{14}C-assimilate recovery in grapefruit ('Foster' pink seedless clone # 1-26-39) 24 h after 1 h of light or dark $^{14}CO_2$ fixation by either fruit, or an adjacent source leaf. Exposure to $^{14}CO_2$ (13.6 uCi l^{-1}) was followed by ambient air for 24h either in a 12:12 light:dark period or in continuous dark.

Date[z]	A single source leaf		Detached fruit	
	Light	Dark	Light	Dark
	(dpm fruit^{-1} x 10^8)			
May 7	2.74 ± 0.75[y]	0.006 ± 0.002	1.41 ± 0.34	0.026 ± 0.001
June 25	0.84 ± 0.27	ND	1.49 ± 0.22	0.032 ± 0.004
July 27	0.41 ± 0.12	ND	2.81 ± 0.82	0.067 ± 0.010
Sept 10	0.26 ± 0.11	ND	0.75 ± 0.20	0.051 ± 0.010
	(µmol C fruit^{-1})[x]			
May 7	8.78 ± 2.33	0.020 ± 0.006	10.78 ± 2.62	0.53 ± 0.02
June 25	2.64 ± 0.88	ND	15.89 ± 2.35	0.64 ± 0.08
July 27	1.29 ± 0.35	ND	36.51 ±11.21	0.99 ± 0.14
Sept 10	0.87 ± 0.37	ND	14.03 ± 3.49	1.97 ± 0.51
	(dpm g^{-1} fruit dry wt x 10^4)			
May 7	369.7± 100.9	0.77 ± 0.11	179.3 ± 40.4	2.70 ± 0.28
June 25	26.4± 10.0	ND	43.5 ± 5.1	1.03 ± 0.07
July 27	9.4± 3.2	ND	60.0 ± 16.6	1.50 ± 0.17
Sept 10	4.5± 1.7	ND	14.6 ± 3.5	0.88 ± 0.13

[z] Fruit were harvested on May 7, June 25, July 27 and Sept. 10, 1986, when approximately 2, 3.5, 5, and 6.5 months old, respectively. The amount recovered from dark $^{14}CO_2$ fixation in leaves was not detectable after June.

[y] Values are means of 4 replications ± SE. Values for source leaves in the light are amounts of leaf ^{14}C-photosynthates translocated to fruit, representing 58.11 ± 4.48%, 41.77 ± 10.56%, 29.16 ± 8.99%, and 32.95 ± 4.80% of total dpm recovered for May, June, July, and September, respectively.

[x] The C contributions were calculated based on measurements of changing specific activity during experiments (see text), and on expected differences in numbers of carbon atoms labeled in products of photosynthesis vs dark $^{14}CO_2$ fixation.

fruit development, when dry weight was increasing most rapidly (Table 3-1). This is consistent with observations in citrus (Kadoya, 1974; Lenz, 1979) and numerous other species (Wardlaw, 1968; Bollard, 1970; Daie, 1985) which show that photosynthetic rates are often greater when fruit are present. In addition, greater proportions of photosynthetic products are typically exported when demands for them are high (Wardlaw, 1968; Bollard, 1970; Daie, 1985).

Fruit $^{14}CO_2$ fixation in the light increased from May to July, as fruit surface area expanded, but decreased thereafter (Table 3-1). Reductions in fruit photosynthesis at later stages of development have also been identified in other citrus fruit (Bean and Todd, 1960; Moreshet and Green, 1980), but the drop observed here substantially preceded color break. Total µmol C derived from 1 h of dark fixation in $^{12}CO_2$ + $^{14}CO_2$ by fruit increased during development, and probably resulted from the larger fruit size.

In all $^{14}CO_2$ assimilation experiments, greatest total dpm per gram fruit dry weight was recovered from very young grapefruit in May. Values gradually decreased during fruit development (Table 3-1).

The relative C contributed to fruit from 1 h CO_2 fixation by various sources was further compared using parameters described in Table 3-2. Calculations included µmol C recovered in fruit, time, bias correction, and the numbers of leaves required to support fruit growth. Results showed that leaves were the primary C source for fruit growth throughout development (Table 3-3). Fruit photosynthesis was also an important source of assimilates (18.9% to 47.3%) after the majority of expansion had occurred. Dark CO_2 fixation by fruit provided only a small amount of C to fruit growth throughout the fruit

Table. 3-2. Parameters utilized for assessments of relative C contributions by various sources to growth of grapefruit.

Sources	umol C recovered in fruit	Time (12h)[z]	Bias correction[y]	# of leaves or fruits[x]	Sum
Leaf[w] (Light)	LL	1	1 to 2	30 to 60	30 to 120
Fruit[v] (Light)	FL	1	0.1 to 1	1	0.1 to 1
Fruit[u] (Dark)	FD	2	1	1	2

[z] Unpublished data (Tomlinson and Koch, 1987) indicate "dark" CO_2 fixation by PEP carboxylase continues in juice sacs in the light.

[y] CO_2 level decreased slowly during the 60 min exposure time due to the large volume of the system relative to leaf size. However, CO_2 levels during some experiments dropped to approximately one half the initial level, potentially decreasing total CO_2 assimilation for the period by the same amount. Light $^{14}CO_2$ fixation by fruit took place in CO_2 levels 8 times greater than ambient air only during May when respiratory rates were very high (not shown).

[x] Research of Shamel and Pomeroy (1934) indicate a minimum of 60 leaves are required to support a single grapefruit, but assimilates from these leaves were also allocated to other parts of the tree. Data in the present study show that demand by fruit would be maximal only during early fruit development when rates of growth were greatest.

[w] Relative contribution of leaf photosynthesis to fruit $= \dfrac{(30 \text{ to } 120) \times LL}{(30 \text{ to } 120)\ LL + FL + 2\ FD}$

[v] Relative contribution of fruit photosynthesis to fruit $= \dfrac{FL}{(30 \text{ to } 120)\ LL + FL + 2FD}$

[u] Relative contribution of dark fruit CO_2 assimilation to fruit $= \dfrac{2\ FD}{(30 \text{ to } 120)\ LL + FL + 2\ FD}$

Table 3-3. The relative overall C contribution by various sources to growth of grapefruit.

Time	Source leaves		Fruit[z]	
	Light	Dark	Light	Dark
	(% of overall C contribution)			
May	95.7 to 98.9	0.01	1.0 to 3.9	0.1 to 0.4
June	82.2 to 94.9	ND[y]	4.8 to 16.5	0.4 to 1.3
July	50.1 to 80.1	ND	18.9 to 47.3	1.0 to 2.6
Sept	59.2 to 85.3	ND	11.5 to 31.8	3.2 to 8.9

[z] Values for CO_2 fixation by fruit should be considered minimal estimates due to the higher internal CO_2 concentration and extensive refixation of respiratory CO_2.

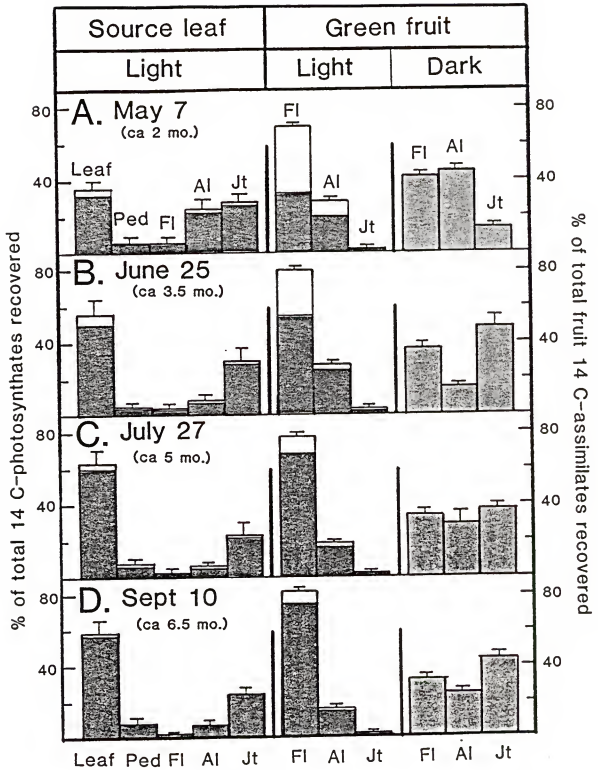
[y] not detectable at later stages.

development. Kriedemann (1968c) suggested that dark CO_2 fixation by apricots could continue contributing to growth of their flesh after fruit chlorophyll content had decreased. Although carbon from 1 h of dark CO_2 fixation by grapefruit increased considerably at later stages of fruit development. It reached a maximum estimate of only 3.2% to 8.9% of total C recovered from various sources (Table 3-3). The suggestion by Bidwell (1983) that dark CO_2 fixation may be an anaplerotic reaction in plant tissues, is consistent with the high percentage recovered in grapefruit 24 h later as organic and amino acids (about 40% for each).

Data in Fig. 3-2 show that in May, when young fruit were accumulating dry weight most rapidly, about 58% of the total ^{14}C-photosynthates from adjacent source leaves was transported into fruit within 24 h. The percent of ^{14}C-photosynthates exported from leaves to fruit decreased during fruit development, as did the overall rate of dry weight increase by fruit. Results are consistent with previous studies which indicate that the presence of fruit can increase the proportion of the photosynthates exported (Bollard, 1970; Kadoya, 1974; Lenz, 1979). The stage of calamondin fruit development has also been found to influence overall allocation of photosynthates within trees (Lenz, 1979).

Distribution of leaf-derived ^{14}C-photosynthates within fruit appeared to change during development in approximate relation to the extent of new dry matter added to individual tissue. Still, juice tissues and albedo shared most of the ^{14}C-assimilates imported from leaves in May, despite nearly equal dry weight gains by flavedo, albedo and juice tissues. In addition, new dry weight added to juice

Fig. 3-2. Developmental changes in partitioning and distribution of ^{14}C-assimilates in grapefruit ('Foster' pink seedless clone # 1-26-39) after exposure of either intact detached fruit or an adjacent source leaf to $^{14}CO_2$ for 1 h and ambient air for 24 h in either a 12:12 light:dark regime or continuous dark. Shaded (■) and open (□) portions of columns indicate respective partitioning between soluble and insoluble (starch and/or cell wall constituents) fractions within a given tissue. Vertical bars at the tops of columns denote the SE of 4 replications for tissue totals. Ped=pedicel, Fl=flavedo, Al=albedo, Jt=juice tissues.


Source leaf
Green fruit
Light
Light
Dark
A. May 7
(ca 2 mo.)
B. June 25
(ca 3.5 mo.)
C. July 27
(ca 5 mo.)
D. Sept 10
(ca 6.5 mo.)
Leaf
Ped
Fl
Al
Jt
80
40
% of total 14 C-photosynthates recovered
% of total fruit 14 C-assimilates recovered
Leaf Ped Fl Al Jt Fl Al Jt Fl Al Jt

tissues demonstrated that they were by far the dominant sink for photosynthates in fruit after June, yet the proportion of leaf ^{14}C-assimilates recovered in juice tissues was greater still. The basis for this difference may have been gain in long-term loss of ^{14}C-photosynthates from peel either through transfer to other tissues or via greater release of respiratory $^{14}CO_2$.

Translocation and distribution of ^{14}C-assimilates resulting from $^{14}CO_2$ fixation by leaves in the dark was similar to that in light, but a greater percentage (about 80%) was retained in leaves.

Distribution of ^{14}C-assimilates within fruit differed depending on the site of $^{14}CO_2$ fixation, its occurrence in light vs. dark, and the stage of fruit development (Fig. 3-2).

Flavedo appears to be able to fix CO_2 either from the external environment or from endogenous respiration (Bean et al., 1963). Photosynthetic refixation of respiratory CO_2 by grapefruit was indicated in the present study by a comparison between CO_2 exchange in light and dark (data not shown). However, 24 h after photosynthesis by intact fruit, the photoassimilates were shown here to remain predominantly in the peel (either pigmented flavedo or white albedo) (Fig. 3-2). More than 60% of the ^{14}C-photosynthates remained in flavedo 24 h after fruit were exposed to $^{14}CO_2$ in the light. Less than 30% of the total ^{14}C-photosynthates moved inward to the albedo, and fewer than 4% were recovered in juice tissues. This is markly different from the situation in soybean and pea, where conservation of respiratory CO_2 contributes to seed growth (Quebedeaux and Chollet, 1975; Atkins et al., 1977).

Distribution of ^{14}C-assimilates in fruit after dark $^{14}CO_2$ fixation

differed substantially from that of photosynthesis in the light (Fig. 3-2). A considerably greater portion of the assimilates labeled during dark CO_2 fixation were localized in juice tissues, and in several instances also the albedo. More radioactivity was recovered in juice tissues than any other fruit part, except in May when juice sacs made up the smallest portion of the fruit. The total percentage of ^{14}C-assimilates in juice tissues increased dramatically after June and remained constant at later stages.

Dark CO_2 fixation can be an anaplerotic reaction for amino and organic acid synthesis and is often associated with growth, pH balance, and energy supply in plants (Latzko and Kelly, 1983). Assimilation of CO_2 by fruit observed here may also be related to the organic acid synthesis in citrus fruits as proposed by Huffaker and Wallace (1959). The developmental timing and extent of dark CO_2 fixation in the present study are consistent with both of the above. The role of dark CO_2 fixation in organic acid synthesis will be examined further in subsequent chapters.

A greater portion of fruit ^{14}C-photosynthates were generally recovered in the ethanol-soluble fraction (primarily sugars, organic acids, amino acids and essential oils) than in the ethanol-insoluble fraction (primarily starch and structural components) (Fig. 3-2). Radioactivity in the ethanol-insoluble fraction was greatest early in fruit development, and might be associated with the rapid increase in dry weight of cell wall constituents during this period. The ratio of ^{14}C-assimilates incorporated into the alcohol-soluble and insoluble fractions of flavedo, albedo and juice tissues were approximately similar to the total proportions of those constituents in the tissues

Table 3-4. Distribution of ethanol-soluble ^{14}C-assimilates in fruit tissues after 1 h of photosynthesis by intact fruit in $^{14}CO_2$, and 24 additional h in ambient air.

Treatments[z]	% of ethanol-soluble 14C-photosynthates recovered[y]			Total dpm recovered
	Flavedo	Albedo	Juice tissues	dpm x 10^8
June				
1h	92.5 ± 1.4	6.0 ± 1.5	1.5 ± 0.2	2.17 ± 0.33
1h + 24h	64.6 ± 3.1	27.2 ± 2.4	8.2 ± 0.9	0.98 ± 0.47
July				
1h	96.0 ± 2.1	2.8 ± 1.4	1.2 ± 0.7	3.14 ± 3.36
1h + 24h	69.7 ± 0.5	23.4 ± 0.8	6.9 ± 0.6	4.74 ± 1.64
Sept				
1h	95.0 ± 0.5	3.9 ± 0.4	1.1 ± 0.1	3.57 ± 0.55
1h + 24h	75.7 ± 1.0	22.3 ± 1.0	2.0 ± 0.2	2.73 ± 0.58

[z] Fruit harvested on June 7, July 8, and Sept. 10, 1985, after approximately 3, 4, 6 months' growth, respectively.

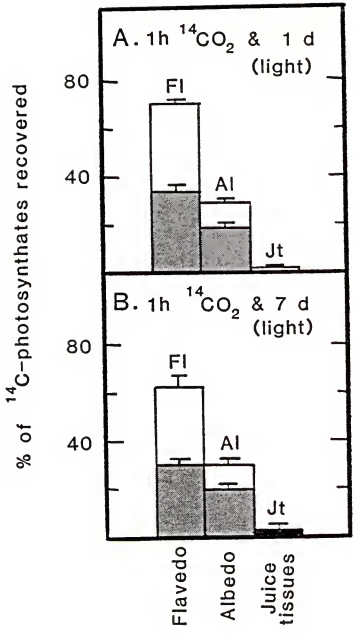
[y] Value are means of 4 replications ± SE.

(Sinclair and Jolliffe, 1960).

Some redistribution of fruit ^{14}C-assimilates from flavedo to interior tissues was indicated by a comparison between distribution 1 h and 24 h after fruit photosynthesis in $^{14}CO_2$ fixation by fruit in light (Table 3-4). Percentages of labeled ethanol-soluble photosynthate recovered in albedo and juice tissues of intact fruit were minimal after 1 h (means of 4.2 % and 1.3 %, respectively), suggesting that little inward movement of labeled assimilates had occurred during this time and/or that rates of exogenous $^{14}CO_2$ fixation by inner tissues were low. Portions transferred to albedo during the subsequent 24 h were considerably greater than those moving into juice tissues. Approximately one quarter of the total was recovered from the inner peel, while, only 0.7% to 2.4 % of the fruit ^{14}C-photosynthates had moved into juice tissues after 24 h (Fig. 3-3).

Even 7 days after $^{14}CO_2$ administration to fruit, a mean of less than 4 % of labeled fruit photosynthates had moved inward to juice sacs (Fig. 3-3). Photosynthesis in fruit also contributed minimally to total carbon balance of juice tissues in lemon and orange (Todd et al., 1961; Moreshet and Green, 1980). Results here also support this conclusion for grapefruit juice sacs, but data from other citrus differ. In calamondin fruit, for example, about 23% of fruit ^{14}C-photosynthates were recovered in juice tissues 7 days after exposure of intact fruit to $^{14}CO_2$ (Chapter V). In satsuma mandarin 25% to 26% of photosynthates labeled by fruit were localized in juice tissues 5 days after exposure to $^{14}CO_2$ (Akao and Tsukahara, 1980a). The contrasting results may be due to structural differences between peel of calamondin, satsuma mandarin and grapefruit, because albedo of

Fig. 3-3. Partitioning and distribution of ^{14}C-photosynthates after exposure of intact, recently-detached grapefruit ('Foster' pink seedless clone # 1-26-39) to $^{14}CO_2$ for 1 h followed by ambient air for 1 day (A) or 7 days (B) in a 12 h light:12 h dark regime. Shaded (■) and open (□) portions of columns indicate respective partitioning between soluble and insoluble (starch and/or cell wall constituents) fractions within a given tissue. Vertical bars at the tops of columns denote the SE of 4 replicatios for tissue totals, and are too small to be pictured where not shown. Similar designations within columns represent the SE for component fractions of each total. Fl=flavedo, Al=albedo, Jt=juice tissues. Total dpm recovered after 1 day and 7 days were 1.41 ($\pm$0.34) X 10^8 and 0.39 ($\pm$0.04) X 10^8, respectively, equivalent to 10.78 $\pm$ 2.62 and 6.92 $\pm$ 0.71 µmol total CO_2 fixed.

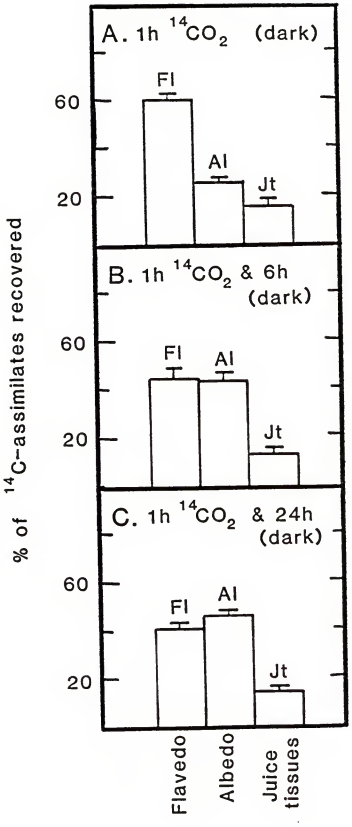

A. 1h $^{14}CO_2$ & 1 d (light)
B. 1h $^{14}CO_2$ & 7 d (light)
80
40
Fl
Al
Jt
% of ^{14}C-photosynthates recovered
Flavedo
Albedo
Juice tissues

the latter comprises a far greater percentage of the total fruit volume.

Inward redistribution of ^{14}C-assimilates initially fixed in green peel of grapefruit could have occurred via entry into the extensive vascular network in the flavedo, but photosynthates arising from leaves presumbly would be unloading from phloem at or near the same sites. Movement could also have proceeded slowly from cell-to-cell as long as a favorable gradient of solutes existed. Sucrose and hexose concentration at this stage of development are compatible with the above interpretation. Another possibility is that of internal diffusion and refixation of respiratory $^{14}CO_2$, favored by extensive air spaces in albedo, and by the heavy cutinization of the flavedo.

Localization of ^{14}C-assimilates 1 h after exposure of fruit to $^{14}CO_2$ in the dark changed only within peel during the subsequent 6 and 24 h (Fig 3-4), indicating that radioactivity recovered in juice tissues was the likely result of carboxylation reactions at the same site. This also implies that ample $^{14}CO_2$ could diffuse from the atmosphere into juice tissues despite the much higher CO_2 concentration reported inside the fruit by Eaks and Ludi (1960). The percentages of ^{14}C-assimilates in albedo increased over the 24 h period after initial fixation (Fig. 3-4). The total recovered dpm did not differ significantly between 1 and 6 h, however (data not shown). Albedo appeared to have the lowest respiratory rate when individual fruit tissues were measured (data not shown). The greater percentage of ^{14}C-assimilates recovered in albedo relative to other fruit tissues after 24 h may partially reflect a lesser loss of ^{14}C-assimilates from respiration and related activity in albedo.

Fig. 3-4. Distribution of ^{14}C-assimilates within young grapefruit ('Foster' pink seedless clone #1-26-39, 2-months old) after exposure of recently-detached fruit to $^{14}CO_2$ (A) for 1 h, followed by (B) ambient air for an additional 6 h or (C) 24 h in continuous dark. Vertical bars denote SE of 4 replications. Fl=flavedo, Al=albedo, Jt=juice tissues. Total mean dpm recovered at 1 h, 1+6 h, and 1+24 h were 255,150 (± 26,010), 140,474 (± 24,873), and 255,717 (± 7,813), respectively.


A. 1h $^{14}CO_2$ (dark)
B. 1h $^{14}CO_2$ & 6h (dark)
C. 1h $^{14}CO_2$ & 24h (dark)
Fl
Al
Jt
60
20
% of ^{14}C-assimilates recovered
Flavedo
Albedo
Juice tissues

Conclusions

Distribution of labeled assimilates after light and dark $^{14}CO_2$ fixation by leaves and fruit was as follows:

1. Exported products of both light and dark $^{14}CO_2$ fixation in leaves were deposited primarily in juice sacs of fruit. More of the assimilates were retained in leaves after dark CO_2 fixation.
2. Fruit photosynthesis gave rise to assimilates which remained primarily in flavedo and albedo 24 h later, thus probably contributing little to growth of juice tissues.
3. ^{14}C-assimilates from dark $^{14}CO_2$ fixation in intact fruit were localized primarily in juice tissues.

An assessment of the relative C contributed by each assimilate source to fruit development at various stages of growth indicated that leaf photosynthesis was most important when fruit were very young. As fruit expanded, the proportion of C contributed by fruit CO_2 fixation either in light or in the dark increased. The potential significance of dark $^{14}CO_2$ assimilation to organic acid synthesis in fruit will be addressed in subsequent work.

CHAPTER IV
ORGANIC ACIDS FROM LIGHT AND DARK CO_2 FIXATION BY LEAVES AND FRUIT OF LOW-ACID CITRUS MUTANTS

Introduction

Levels of organic acid in fruit can dramatically affect flavor attributes such as sourness (Ulrich, 1970). The sugar to acid ratio is also an important criterion for determining the maturity of citrus and other fruits (Ulrich, 1970). Citric and malic acid, which are the main organic acids in citrus fruit, are important to many aspects of plant metabolism (Raven and Smith, 1976; Edwards and Huber, 1981; Lance and Rustin, 1984; Bown, 1985). The involvements of organic acids in photosynthesis, pH balance, and nitrogen assimilation are well known. Fleshy fruits are a rich source of organic acids (Ulrich, 1970), but the mechanism and control of synthesis and accumulation remain unclear. Citrus fruit acidity is affected by many factors. Organic acid content varies widely among fruit species (Thimann and Bonner, 1950; Ulrich, 1970), between fruit on the same tree (Sinclair, 1984), and even at different locations within the same fruit (Ting, 1969). A significantly higher ratio of citric:malic acid occurs in sour types of citrus fruit, with the reverse evident in less sour fruit. Citric acid predominates in juice tissues but malic acid is greater in the peel. The acid content in citrus fruit can also be affected by climate (Reuther, 1973) and horticultural practices such as irrigation (Koo and McCornack, 1965; Gilfillan et al., 1976), fertilization (Koo and

McCornack, 1965), and arsenical sprays (Wilson, 1983).

Three hypotheses have been proposed to account for the possible origin(s) of the mechanism of organic acids accumulation in citrus fruit:

1. Organic acids may be produced in fruit by metabolism of photosynthates transported from leaves (Varma and Ramakrishnan, 1956; Ramakrishnan, 1971).

2. Organic acids may be transported unaltered, directly from leaves or roots (as reviewed by Ulrich, 1970).

3. Organic acids may be formed in fruit by fixation of respiratory or atmospheric CO_2 via phosphoenolpyruvate(PEP) carboxylase (Huffaker and Wallace, 1959) and/or metabolism of carbohydrates produced during C_3 photosynthesis in fruit.

Reciprocal grafting between fruit of sweet and sour lemons, and their respective leafy branches, suggested that a primary mechanism affecting organic acid accumulation was probably localized in fruit rather than leaves (Erickson, 1957). Organic acid synthesis in the fruit tissue presumably would occur through the combined operation of the glycolytic pathway and TCA cycle (Varma and Ramakrishnan, 1956; Ramakrishnan, 1971), utilizing carbohydrates which might arise either from leaf or fruit photosynthesis (Bartholomew and Reed, 1948). No direct evidence has been provided to support this hypothesis, although it is favored by researchers studying grape (Ruffner et al., 1983).

Most organic acids found in fruit have been detected in exudates from phloem. The type of organic acids varies with different plant species. However, organic acids are only a minor component in phloem exudates (Ziegler, 1975) and initially seem insufficient to explain

the large amount acid accumulated in citrus fruit.

The extent to which products of CO_2 fixation in fruit are partitioned into organic acids is still unknown. Dark CO_2 fixation could be an anaplerotic carboxylation reaction for amino acid and lipid synthesis and may be related to a metabolic pH balance in cells (Davies, 1973; Bidwell, 1983). Huffaker and Wallace (1959) suggested that dark CO_2 fixation was related to acid accumulation in citrus fruit and that PEP carboxylase was a key enzyme for CO_2 fixation. More assimilates were recovered as organic acids after dark CO_2 fixation than in light after photosynthesis by lemon fruit (Bean and Todd, 1960). However, the relationship between amounts of dark CO_2 fixation and fruit acidity conflicted when different methods or parts of fruit were used (Clark and Wallace, 1963; Bogin and Wallace, 1966a). The actual source and pathway of organic acid synthesis in citrus fruit are still unclear.

The objectives of this study were to clarify the role of light and dark CO_2 fixation in organic acid accumulation by citrus fruit and to quantify the amount of organic acid derived from leaves, peel and juice tissues throughout fruit development. Two grapefruit clones were also compared, which are known to differ from one another in that levels of fruit acid are normal in one and low in the other (Hearn, 1986).

Materials and Methods

Normal- and low-acid grapefruit clones (seedless mutation # 1-26-39, and 1-26-51, respectively) were derived from 'Foster' pink grapefruit (Citrus paradisi, Macf.) by bud irradiation (Hearn, 1986).

Experimental trees were grown in Lake Wales, FL, and were 4-years old on 'Savage' sour orange or sour orange rootstocks. Fruits and/or branches with source leaves adjacent the fruit were randomly harvested from 1 to 2 m above ground on the exterior of the canopy. Samples were collected on May 7, June 25, July 27 and September 10, at about 40-day intervals, after 2, 3.5, 5 and 6.5 months' growth, respectively. Several samples from the same trees were used for different treatments and so that one tree constituted a replication. Four replications were used for each experiment. Stems were immediately immersed in water and transported to Gainesville for experiments.

At each developmental stage, fruits or leaves adjacent to them were enclosed in a cuvette and exposed to $^{14}CO_2$ (13.6 μCi l^{-1}) for 1 h in light followed by ambient air for 24 h (12 h:12 h light:dark photoperiod). Cuvettes were completely sealed with a foil covering for dark treatments. Levels of CO_2 were monitored during experiments by circulating air through an infrared gas analyzer (IRGA) (Model 865, Beckman Instruments Inc., Fullerton, CA). Data were used to calculate mean specific activities of $^{14}CO_2$ during periods when release of respiratory CO_2 increased total levels. Temperature was maintained at 28°C and photosynthetic photon flux density at 800 to 1000 μmol m^{-2} s^{-1}. Light was provided by a Lucalux lamp (Lu 400, General Electric Co. Cleveland, OH).

Each experiment was terminated by separating tissues into flavedo (pigmented portion of peel), albedo (non-pigmented portion of peel) and juice tissues. Samples were frozen in liquid nitrogen, extracted in 80% (v/v) ethanol, and the radioactivity in each was quantified by

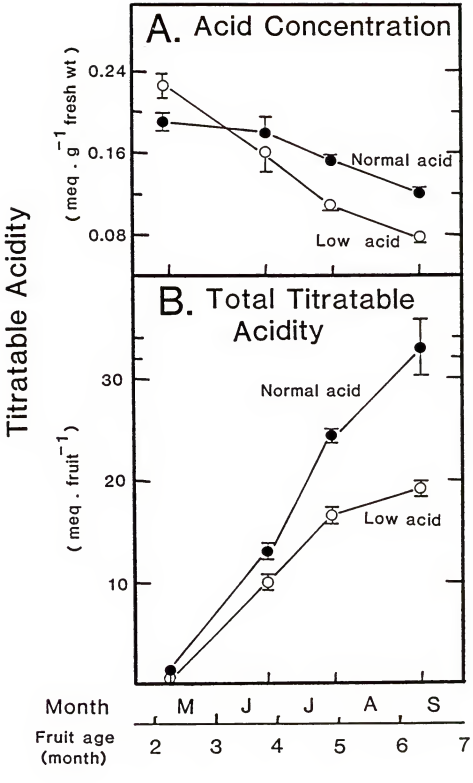
liquid scintillation (LKB, Gaithersburg, MD). The ethanol-soluble fraction was measured by pipetting 0.1 ml of sample into 15 ml scintillation cocktail (Scinti Verse II, Fisher Scientific Co., Fair Lawn, NJ). The ethanol-insoluble fraction was measured by suspending a known portion of the sample in scintilation cocktail. Quench curves were established for materials utilized, but were generally not needed for conversion of cpm to dpm.

The ethanol-soluble fraction was further fractioned with ion-exchange resin (Dowex 1-x8) using a modified procedure of Atkins and Canvin (1971). Organic acids were eluted from the anion resin with 30 ml 6 N formic acid and further analyzed by HPLC (Bio-Rad, Richmond, CA). Samples were first passed through a C18 Sep-Pak cartridge (Water's Associates, Milford, MA) and 0.45 μ filters. A 20 ul sample was then injected and separated using an ion exchange column (Aminex HPX 87H, 300 X 7.8 mm) at 65°C. The mobile phase was 0.01N H_2SO_4 at a flow rate of 0.4 ml min^{-1}. The ^{14}C-citric and malic acid fractions were collected (Gilson International Co., Middleton, WI) and measured by liquid scintillation spectroscopy as mentioned above.

Results

Acid concentration decreased gradually during growth of grapefruit with normal acid levels, but more rapidly in fruit of the low acid clones (Fig. 4-1A). Early in development, juice tissues of the low acid clone had a significantly higher acid content than did those of its normal acid counterpart. The same result was observed the previous season (not shown). Differences in acid levels were most evident in juice tissues, but also in flavedo (pigmented portion of peel),

Fig. 4-1. The acid concentration (A) and total titratable acidity (B) in juice tissues from fruit of normal and low acid grapefruit clones in May, June, July and September, at about 2, 3.5, 5, and 6.5 months of development. Vertical bars denote SE of 4 replications, and are smaller than symbols where not pictured.


Titratable Acidity
A. Acid Concentration
(meq . g -1 fresh wt)
0.24
0.16
0.08
Normal acid
Low acid
B. Total Titratable Acidity
(meq . fruit -1)
30
20
10
Normal acid
Low acid
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

followed by albedo (non-pigmented portion of peel) (data not shown). The more rapid drop in acidity, however, brought levels from the low acid clone to below those of the normal acid clone after June (Fig. 4-1A). At 3.5 months of development or after, total titratable acidity of juice per fruit (Fig. 4-1B) was also significantly lower in the clone known to have less acid at commercial harvest.

Further analysis by HPLC showed that citric and malic acids were the primary organic acids in juice tissues of both grapefruit clones (Table 4-1). Values are comparable to those previously reported for grapefruit with normal levels of total acidity (Monselise and Galily, 1979). The ratio of citrate to malate was lowest early in development and increased rapidly after June. In September, citric acid levels were 14- and 9-fold greater than those of malic acid for fruit of normal and low acid grapefruit clones, respectively.

Quantification of label in ^{14}C-organic acids recovered from whole fruit indicated that organic acid synthesis continued throughout development (Fig. 4-2). About 1% or less of the labeled photosynthates translocated to fruit from an adjacent leaf had been converted to organic acids of juice tissues 24 h after initial $^{14}CO_2$ fixation (Fig. 4-2A). Values were greater when organic acids were expressed as percentages of ^{14}C-assimilates in juice tissues alone (Fig. 4-2B). In both instances, the extent of organic acid formation from imported leaf photosynthates dropped by 50% or more between the 2nd and 4th months of growth. No significant differences were noted between normal vs. low acid fruit.

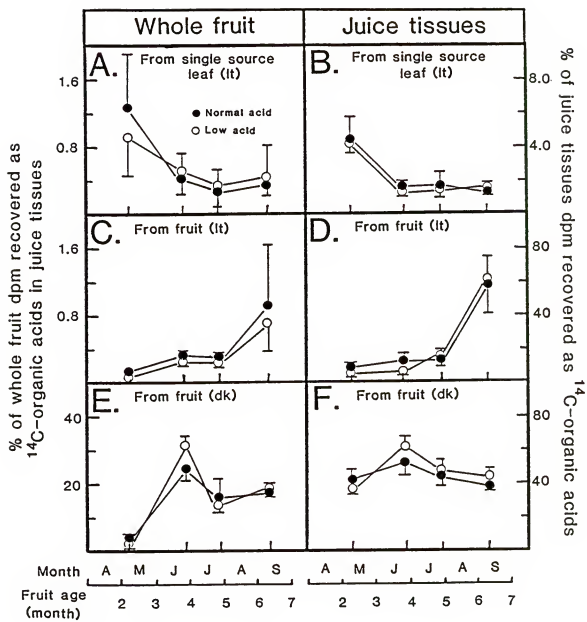
In contrast to labeled photosynthates from adjacent leaves, those derived from fruit photosynthesis were recovered to an increasing

Table 4-1. The ratio of citric to malic acid in juice tissues during development of fruit on normal and low acid grapefruit clones.

	Ratio of citrate/malate (Citric acid/malic acid)			
	May	June	July	Sept
Normal Acid Mutant	_	10.6 ± 1.1[z]	10.0 ± 0.7	13.8 ± 1.2
Low Acid Mutant	_	9.4 ± 0.7	9.2 ± 0.6	9.4 ± 0.5

[z] Means of 6 replications ± SE (6 fruits from 6 different trees) for June, July, and September.

Fig. 4-2. Developmental changes in the percentage of 14C-assimilates partitioned into organic acids of juice tissues 24 h after a 1 h period of photosynthesis in $^{14}CO_2$ by an adjacent leaf (A, B), by an intact, detached fruit (C, D) or 1h of dark $^{14}CO_2$ fixation by an intact fruit (E, F) from normal and low acid grapefruit clones. Figures on the left (A,C,E) denote ^{14}C-organic acid percentages of total ^{14}C-assimilates translocated to, or retained in fruit. Those on the right (B,D,F) show ^{14}C-organic acids as percentages of total ^{14}C-assimilates translocated to, or retained in juice tissues. Experiments were conducted using fruit approximately 2, 3.5, 5, and 6.5 months of growth. Note the differences in percentage units among the figures. Vertical bars denote the SE of 4 replications, but are present only where larger than symbols.

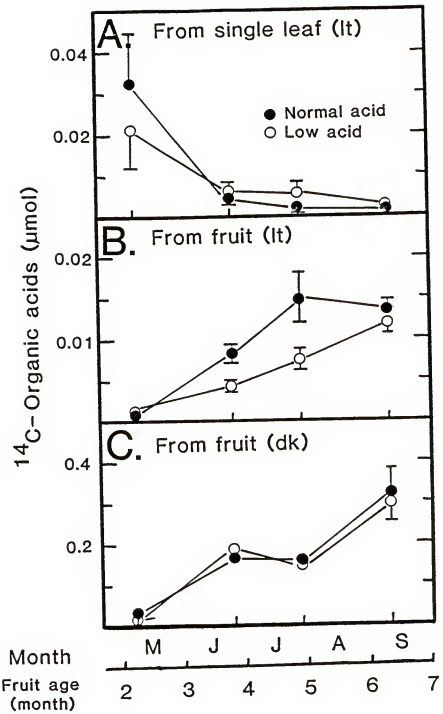

Whole fruit
Juice tissues
A. From single source leaf (lt)
B. From single source leaf (lt)
Normal acid
Low acid
C. From fruit (lt)
D. From fruit (lt)
E. From fruit (dk)
F. From fruit (dk)
% of whole fruit dpm recovered as 14C-organic acids in juice tissues
% of juice tissues dpm recovered as 14C-organic acids
1.6
0.8
8.0
4.0
80
40
20
Month
A M J J A S
Fruit age (month)
2 3 4 5 6 7

extent as organic acids during fruit development (Fig. 4-2 C and D). This was true even when only the ^{14}C-assimilates in juice tissues were considered (Fig. 4-2D). More than 50% of the fruit ^{14}C-photosynthates localized in juice tissues were organic acids at later stages of fruit growth (September) (Fig. 4-2D). Differences between fruit of normal- and low-acid lines were not significant.

A greater percentage of ^{14}C-assimilates were partitioned into organic acids after dark CO_2 fixation by fruit than after photosynthesis by either leaves or fruit (note units in Fig. 4-2 E and F). Values ranged from 40% to 60% and were highest in fruit about 3.5 months old. Percentages may have been affected by the extent of amino acid synthesis from organic acid products of dark $^{14}CO_2$ fixation. Again, fruit of the two grapefruit clones did not differ significantly.

Figure 4-3 shows the amount of organic acids synthesized in juice tissues from assimilates produced during 1 h of photosynthesis by a single, adjacent leaf, by the intact fruit, or by dark CO_2 fixation in the fruit. These data were used to estimate the extent of total, overall organic acids arising directly or idirectly from the sources described in Table 4-2. Equations and parameters employed were those discussed in Chapter III (Table 3-2). Calculations considered differences in overall input from leaves relative to changes in CO_2 levels during experiments, possible and reported numbers of leaves involved, the portion of a 24 h day in which a given process would be expected to occur. Results appeared that the major sources of organic acid formation during early fruit growth were photosynthates translocated from adjacent leaves and dark CO_2 fixation in fruit

Fig. 4-3. The µmol of organic acids contributed to juice tissues 24 h after a 1 h period of CO_2 fixation by either in an adjacent leaf in the light (A), fruit in the light (B), or fruit in the dark (C). $^{14}CO_2$ fixation (Fig. 4-2), from expected differences in the numbers of C atoms labeled during photosynthesis vs. dark $^{14}CO_2$ fixation, and from changes in specific activity of atmospheric $^{14}CO_2$ determined from infrared gas analysis of total CO_2 levels during exposure. Vertical bars denote the SE of 4 replications, which is shown only where larger than the symbols used.


A. From single leaf (lt)
0.04
0.02
Normal acid
Low acid
B. From fruit (lt)
0.02
0.01
C. From fruit (dk)
0.4
0.2
^{14}C- Organic acids (µmol)
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

(Table 4-2). Later in fruit development, however, dark CO_2 fixation by fruit increased in importance and became one of the major sources of organic acids. In contrast, fruit photosynthesis appears to play only a minor role in organic acid synthesis.

Discussion

Decrease of organic acid concentration toward maturity has been widely reported in citrus fruit (Ting and Vines, 1966; Erickson, 1968; Sinclair, 1984). A comparison of Fig. 4-1A to B indicates that decreases in acidity are due to fruit expansion, and that total organic acid accumulation occurs continuously throughout fruit development. It is unclear why low acid fruit accumulate organic acids less rapidly, especially after the 5th month of fruit development.

Results confirmed that citric and malic acids accounted for most of the organic acids in juice tissues of grapefruit (Monselise and Galily, 1979), but the ratio of citrate to malate was much greater throughout development. Differences between fruit of the two clones were singnificant in September (Table 4-1).

The percentages of ^{14}C-assimilates partitioned into organic acids were significantly different between various sources (Fig. 4-2). This may be associated with the function of different sources in fruit growth (especially when compared with Chapter III). About 40% to 60 % of the assimilates derived from dark CO_2 fixation by juice tissues remained as organic acids 24 h later (Fig. 4-2). This is similar to percentages reported by previous researchers (Bean and Todd, 1960; Young and Biale, 1968).

The initial product of dark CO_2 fixation is oxaloacetate (OAA),

Table 4-2. The contribution of various sources to organic acids in juice tissues of normal and low acid grapefruit during fruit development.

Time	Source leaves		Fruit[z]	
	Light	Dark	Light	Dark
	(% of contribution to organic acids)			
Normal acid				
May	94.0 to 98.4	0.1	0.02 to 0.06	1.5 to 5.8
June	30.9 to 64.1	ND[y]	0.9 to 1.8	35.0 to 67.3
July	18.0 to 46.7	ND	2.5 to 3.9	50.8 to 78.2
Sept	6.6 to 22.0	ND	1.6 to 1.9	76.4 to 91.5
Low acid				
May	93.8 to 98.4	0.1	0.03 to 0.1	1.6 to 6.0
June	34.2 to 67.5	ND	0.4 to 0.8	32.1 to 65.0
July	36.4 to 69.6	ND	0.8 to 1.7	29.6 to 62.0
Sept	12.4 to 36.2	ND	1.3 to 1.8	62.6 to 85.8

[z] Values estimated from $^{14}CO_2$ fixation by fruit represent minimal estimates due to high internal CO_2 levels and extensive refixation of respiratory CO_2.

[y] not detected at later stages.

which is converted immediately to malate (Latzko and Kelly, 1983). Less than 8 minutes were required to turn over malate produced during dark CO_2 fixation in tomato fruit, possibly reflecting the amount present in the cytoplasm (Farineau and Larval-Martin, 1977). Turnover is 157 minutes, however, when malate is stored in vacuoles of root cells (Ting and Dugger, 1966). In their experiments with tomato, Farineau and Laval-Martin (1977) found that 50% of ^{14}C-3-malate infiltrated into tomato flesh in the dark was recovered in C_6 acids (citrate and isocitrate). Malate can apparently turnover rapidly in plant cells during anaplerotic dark CO_2 fixation. In the present study, a ratio of 1.3 for labeled citrate/malate was observed in juice tissues after dark $^{14}CO_2$ fixation by fruit in July. This is considerably less than the ratio of total citrate to malate found in juice tissues (from 9:1 to 14:1). A rapid and highly labeled CO_2 release after dark $^{14}CO_2$ fixation has been reported by Splittstoesser (1966). Therefore, the more extensive label in malate may be due to a slower turnover of acid when stored in vacuoles, or a continuous refixation of respiratory $^{14}CO_2$ released. Together these two acids accounted for about 90% of the of label in the anion fraction.

Although dark CO_2 fixation appears to be a major source of organic acids in grapefruit after 3 months' growth, leaf photosynthates still play an important role by serving as substrates for synthesis of phosphoenolpyruvate (PEP), precursor of the dark CO_2 fixation reaction. Dark CO_2 fixation by leaves was shown not to be a significant source of fruit organic acids.

The mechanism of biosynthesis and sources of organic acids in citrus fruit have been debated for many years (Bartholomew and Reed,

1948; Varma and Ramakrishnan, 1956; Huffaker and Wallace, 1959; Buslig, 1968; Ulrich, 1970; Ramakrishnan, 1971; Kubota and Akao, 1973a; Akao and Kubota, 1978b; Akao and Tsukahara, 1980b) and several hypotheses have been suggested. Results of the present study indicate that leaf photosynthates are the primary source of organic acids in very young citrus fruit (Table 4-2) depite the small amount partitioned into fruit acids from individual leaves (Fig. 4-2 and 4-3). Fruits receive photosynthates from numerous leaves rather than one, and about 60 leaves are required to support normal growth of a single grapefruit (Shamel and Pomeroy, 1934).

Calculations based on Fig. 4-3 (Table 4-2) indicated leaf photosynthates were the predominant source of organic acids during early fruit growth, but decreased rapidly as dark CO_2 fixation become a more important contributor of organic acids at later stages. Calculated values for total organic acid synthesis after light fixation of $^{14}CO_2 + {}^{12}CO_2$ by intact fruit represent minimal amounts. Dilution of atmospheric $^{14}CO_2$ by unlabeled, respiratory CO_2 was measured and accounted for, but not refixation of unlabeled CO_2 retained within tissues. The same holds true for both light and dark CO_2 fixation by fruit.

Conclusions

Acid concentration decreased and total acidity increased continously throughout development of fruit from both normal- and low-acid grapefruit clones, but values were significantly less in grapefruit from the low-acid clone after 5 or more months' growth. In more mature fruit, the citrate to malate ratio was also significantly

less in the low- vs. high-acid fruit, 9:1 and 14:1, respectively.

Significant differences were not evident in amounts of ^{14}C-assimilates partitioned into organic acids in fruit of normal- and low-acid grapefruit clones, but may have resulted from the relatively short initial exposure time (1 h) compared to the duration of fruit development. About 90% of anion fraction of ^{14}C-assimilates recovered from dark $^{14}CO_2$ fixation in juice tissues of 5-month-old fruit was citrate and malate. The ratio of labeled citrate to malate was far less than that of total levels in juice tissues, possibly due to slow turnover of malate in vacuoles or continuous refixation of $^{14}CO_2$ in the dark.

Leaves were the primary source of assimilates for organic acid formation early in fruit development. However, dark CO_2 fixation by fruit became a major, and perhaps predominant source of organic acids later in development, despite of its relatively minor contribution to total C.

CHAPTER V
EFFECTS OF ARSENATE ON ACIDITY AND LONG-TERM ASSIMILATE PARTITIONING IN CALAMONDIN (Citrus madurensis Lour.)

Introduction

The acid level of citrus juice tissue decreases during development, and is important to both quality of fruit and timing of early harvests. A specific soluble solids/acid ratio is required before fruit can be picked, and neither occur until acidity drops below a level considered acceptable relative to total sugars. If this occurs early in the season, a higher price may be obtained for fruit. It has long been known that arsenate-based insecticides will significantly decrease acidity of some maturing citrus fruit. Fruit on sprayed trees can be harvested weeks earlier (Wilson, 1983).

It has also been shown that arsenate is the active agent in this decrease of fruit acidity (reviewed by Wilson, 1983; Rice et al., 1985). Initially, it was thought that reactions affecting acid accumulation might be located within the citrus fruit itself (Erickson, 1957). However, natural background levels of arsenate were unaffected in juice sacs at any time after trees were sprayed. This result suggested the possibility that the primary effects of arsenate spray on fruit acidity might be elicited within the leaves (Rice et al., 1985). Other studies, however, indicated that the arsenate concentration in peel was higher after arsenate treatment (Gilfillan et al., 1976). The possible effects of a slight rise in arsenate

concentration in fruit peel cannot be excluded.

It has not been determined which site of arsenate action results in decreased acidity of citrus fruit, but the question has generated considerable interest over the years. Vines and Oberbacher (1965) suggested that the action of arsenate as an uncoupler of phosphorylation might be associated with the decrease of acidity. Oxidation of pyruvate and α-ketoglutarate, the intermediates of respiration, are also influenced by arsenate (Beevers et al., 1966), while aconitase, also a key enzyme in acid metabolism, is unaffected (Dickman, 1961). Arsenate must be present at a given site to have these effects, however, and at concentrations many times greater than the non- or rarely-detectable amounts in fruit. The possibility of arsenate involvement in metabolism mentioned above is therefore unlikely in citrus juice tissues.

Arsenate has been found to increase respiration of grapefruit leaves and this in turn may be associated with the decreased acidity (Miller et al., 1933). Experiments with isolated pea chloroplasts also indicated that arsenite affected light modulation and photosynthetic induction by interferring with light activation of enzymes in the reductive pentosephosphate cycle (Marques and Anderson, 1986). An additional possibility is arsenate competition with Pi and its influence on the triose P carrier of the chloroplast envelope. This could affect both partitioning and export of photosynthatic products. The effects of arsenate on actively growing leaves are evident immediately in fruit and may persist for about 18 months (Miller et al., 1933). This is not inconsistent with leaf effects on fruit, because photosynthetic products move primarily from leaves to nearby

fruit. The influence of arsenate on decreased fruit acidity is not broadly systemic, however, because only fruit from the sprayed portion of trees were found to have less acid juice (Miller et al., 1933). The action site of arsenate and its relation with acidity reduction therefore remains unclear.

Buslig et al. (1971) reported that calamondin trees provide excellent material for screening the acidity reduction potential of various chemicals. These trees will also flower and fruit when very small, so allow greenhouse and laboratory studies to be conducted on mature, bearing citrus trees. The purposes of experiments described here are 1) to compare the effects of arsenate on fruit acidity when applied to individual fruit versus whole plants, and 2) to compare assimilate partitioning in arsenated and non-arsenated calamondin trees. In this way, one can determine to what extent leaf and fruit sources of organic acid are influenced by arsenate treatment.

Materials and Methods

Calamondin (Citrus madurensis, Lour.) fruit, were used in these studies not only because trees are small, but also because they flower and fruit continuously (Hodgson, 1967). Plants were grown in 15 cm plastic containers (about 2.25 liters) in a greenhouse, and were about 2-years old and 80 cm tall when used. For the first group of experiments, plants were sprayed with arsenate ($1.92\ g\ l^{-1}$) when fruits were one month old, as described by Deszyck and Ting (1958). Four plants were used for each treatment, and 4 fruits were harvested for measurement of juice acidity when they were 3- and 6-months old. The effect of arsenate on whole plants, was compared to treatment of

fruit alone by painting 2-month-old fruit with the same concentration of arsenate as applied to the whole tree. Five fruits were treated on each of 3 plants, and another 3 trees were used for full-spray treatments. Fruit acidity was determined by titration of juice from fruit harvested 3 months after arsenation.

The second group of experiments compared $^{14}CO_2$ assimilation, partitioning, and allocation by leaves of fruit of arsenated and non-arsenated trees. Leaves were exposed to $^{14}CO_2$ in the light and fruit $^{14}CO_2$ fixation was studied in both light and dark. Fruits were about 3 months old and the fully expanded leaves adjacent to them were selected as source leaves for $^{14}CO_2$ assimilation. Either fruits or adjacent leaves were enclosed in a cuvette and exposed to $^{14}CO_2$ (16.0 $\mu Ci\ l^{-1}$) for 1 h followed by ambient air for 7 days. During treatments, temperature was maintained at 28°C and photosynthetic photon flux density at 800 to 1000 $\mu mol\ m^{-2}\ s^{-1}$. Light was provided by a Lucalox lamp (General Electric Co., Cleveland, OH). Cuvettes were completely sealed with foil for $^{14}CO_2$ exposure in the dark. Plants were transferred to a greenhouse the day following treatment.

Seven days after this exposure to $^{14}CO_2$ fixation, source leaves, other adjacent leaves, and fruit were harvested. Fruit were further dissected into peel and juice tissues. Samples were boiled in 80% (v/v) ethanol and homogenized using a Polytron (Brinkmann Instruments, Westbury, N.Y.). Ethanol-soluble and -insoluble fractions were separated by filtration in a Buchner funnel. Radioactivity in each sample was quantified by liquid scintillation (LKB, Gaithersburg, MD). The ethanol-soluble fraction was measured by pipetting 0.1 ml sample into 15 ml scintillation cocktail (Scinti Verse II, Fisher Scientific

Co., Fair Lawn, NJ). The ethanol-insoluble fraction was measured by suspending the sample in scintillation cocktail. Ethanol-soluble assimilates were further fractioned with ion-exchange resin (Dowex 1-X8) (Sigma, St. Louis, MO) by a modification of the procedure of Atkins and Canvin (1971). The organic acid fraction was eluted from the anionic resin with 30 ml 6N formic acid.

Results and Discussion

Decreases of acidity by arsenate treatment were significant in one but not two experiments (Table 5-1). The time of arsenate application might account for the different results. Arsenate treatments did not decrease mean acid levels in calamondin juice tissues when the fruit were arsenated in January and March (Table 5-1, Exp. I). Response after both treatments were also less dramatic than those previously reported by Buslig et al. (1971) who recommended calamondin as a plant for screening chemicals for acid reduction potential. Decreased acidity of citrus fruit appears to be a relatively consistent response to whole-tree arsenate treatment, however, and has been identified in grapefruit, orange, satsuma mandarin and calamondin (Buslig et al., 1971; Akao and Kubota, 1978a; Wilson, 1983).

Results here show that arsenate in June significantly decreased the acidity of calamondin fruit even if only the fruits were treated (Table 5-1, Exp. II). Differences between fruit and whole tree treatments were not significant. Therefore, the decrease of acidity in citrus fruit can potentially be brought about by the action of arsenate in the fruit alone. Whole-tree sprays are typically applied shortly after flowering, however, when fruit are so small that

Table 5-1. Arsenate treatments and acidity (meq g^{-1} fresh wt) of calamondin fruit.

Exp. I.[z]	Arsenated	Non-arsenated
	(meq g^{-1} fresh wt)	
Young fruit	0.94 ± 0.04	1.04 ± 0.09
Mature fruit	0.91 ± 0.07	0.94 ± 0.07

Exp. II.[y]	Arsenated		Non-arsenated
	Fruit only	Whole plant	
	(meq g^{-1} fresh wt)		
	1.09 ± 0.02	1.07 ± 0.06	1.17 ± 0.02

[z] Trees were arsenated when fruit were about 1-month old. Samples were taken on May 19, 1987, when young fruit were about 3-months old and mature fruit about 6 months old. Each value is the mean of measurements from 4 trees, with 4 fruit samples from each.

[y] Trees were arsenated on June 3, 1987, when fruit were about 2 months old, and harvested on Aug. 12, 1987, when fruit were about 5 months old. Each value is the mean of measurements from 3 trees, with 3 fruit sampled from each.

arsenate interception would presumably be very small. Also arsenate residue in grapefruit juice sacs is negligible (Rice et al., 1985) and would therefore appear unlikely to be involved in the decrease of fruit acidity. Still, in some instances arsenate has been found in peel of sprayed trees (Gilfillan et al., 1976). The peel may therefore have some effects on acid accumulation in juice sacs, at least in calamondin fruit where the outer tissues (especially albedo) are thinner than those of most other citrus fruit.

The relative amounts of ^{14}C-assimilates arising from different plant parts and later recovered in calamondin fruit (Table 5-2) were similar to those found previously in grapefruit (Chapter IV). Photosynthesis in single leaves of either arsenated or non-arsenated trees were the largest source of total ^{12}C- plus ^{14}C-assimilates in calamondin fruit, followed by photosynthesis in fruit. Data in Table 5-2 also show that arsenate treatment of calamondin trees had no significant effect on amounts of leaf or fruit photosynthates recovered from fruit 1 week after assimilation. In contrast, significantly more ^{14}C-assimilates were localized in fruit of arsenated trees 1 week after $^{14}CO_2$ fixation by fruit in the dark. In other experiments, it appeared that dark CO_2 fixation via phosphoenolpyruvate carboxylase was a major source of organic acids but the control point of organic acid accumulation seemed more likely to be associated with citrate synthase-aconitase (Chapter IV and VI). At the same time, the sum total of assimilates derived from dark CO_2 fixation in fruit is extremely small when compared to amounts arising from photosynthesis in green fruit tissues or a single leaf (Table 5-2B).

Table 5-2. Total dpm and umol recovered from 3-month old calamondin fruit 7 days after a 1 h exposure of either leaves or fruit to $^{14}CO_2$ in light or dark.

A. Total dpm recovered from fruit

	Single leaf in light (dpm x 10^7)	Fruit in light (dpm x 10^7)	Fruit in dark (dpm x 10^5)
Arsenate	7.018 ± 1.196	2.838 ± 0.394	6.123 ± 1.562
Non-arsenated	5.350 ± 1.080	3.237 ± 0.252	3.839 ± 0.422

B. Total exogenous $^{12}C+^{14}C$ assimilated as CO_2 and subsequently recovered in fruit[z]

	Single leaf in light (μmol)	Fruit in light (μmol)	Fruit in dark (μmol)
Arsenate	4.509 ± 0.768	1.666 ± 0.231	0.058 ± 0.015
Non-arsenated	3.437 ± 0.694	1.901 ± 0.148	0.036 ± 0.004

[z] Values are calculated on the basis of measured changes in specific activity of CO_2 + $^{14}CO_2$ during the assimilation period, and expected numbers of C atoms labeled in products of photosynthesis vs. dark $^{14}CO_2$ fixation.

When photosynthate distribution was compared 7 days after leaf $^{14}CO_2$ fixation in the light, a significantly smaller percentage of the total ^{14}C-assimilates was recovered in the insoluble fraction of leaves from arsenated trees, and more of the ^{14}C-photosynthates were translocated to fruit (Table 5-3). More of the leaf-derived ^{14}C-photosynthates were localized in the peel of arsenate treated plants vs. non-arsenated plants. A larger portion of these labeled assimilates was also present in the ethanol-insoluble fraction (predominantly starch and structural constituents) of both juice tissues and peel of fruit from arsenated trees. The amount of ^{14}C-photosynthates from leaves recovered in juice tissues were not significantly different between these two treatments. Differences in distribution might be due to either altered photosynthate partitioning and translocation from leaves, or greater rates of respiratory $^{14}CO_2$ loss from leaves of arsenated plants. Both may be involved. Higher respiratory rates have been found in leaves of arsenate-treated grapefruit (Miller et al., 1933).

Partitioning and distribution of ^{14}C-photosynthates 7 days after fruit CO_2 fixation in light were similar in fruit from arsenated and non-arsenated trees (Table 5-3). Peel retained a greater portion of ^{14}C-assimilates than it had acquired after either photosynthesis by leaves or dark CO_2 fixation in fruit. A larger fraction of total labeled assimilates was also recovered as ethanol-insoluble compounds after fruit vs. leaf photosynthesis. About 23% of the fruit ^{14}C-photosynthates were recovered in juice tissues of fruit from both arsenated and non-arsenated trees. Akao and Tsukahara (1980a) also found about 25% of the ^{14}C-photosynthates were in juice tissues 5 days

Table 5-3. Assimilate distribution among tissues of arsenated and non-arsenated calamondin trees 7 days after a 1 h exposure of either leaves or fruits[z] to $^{14}CO_2$ in light or dark.

Treatments		Source leaf and adjacent leaves	Peel	Juice tissues
		(% of total recovered ^{14}C)		
Single leaf (Light)				
Arsenated	S[y]	19.13 ± 1.69	11.71 ± 0.81	32.72 ± 2.39
	I	4.91 ± 0.32	17.44 ± 2.36	14.09 ± 0.79
Non-arsenated	S	23.83 ± 1.41	9.99 ± 1.18	34.58 ± 2.97
	I	10.83 ± 2.08	11.79 ± 2.27	8.97 ± 0.84
Fruit (Light)				
Arsenated	S	-	33.44 ± 1.52	18.76 ± 4.54
	I	-	42.23 ± 6.68	5.35 ± 1.34
Non-arsenated	S	-	32.69 ± 1.50	19.75 ± 1.41
	I	-	43.35 ± 2.11	3.97 ± 0.71
Fruit (Dark)				
Arsenated	S	-	13.98 ± 4.52	68.29 ± 9.65
	I	-	12.54 ± 5.75	4.16 ± 0.44
Non-arsenated	S	-	12.38 ± 0.79	72.18 ± 2.95
	I	-	8.59 ± 1.50	3.46 ± 0.42

[z] Fruit were about 3 months old.

[y] S= ethanol-soluble fraction, including mono- and dissacharides, organic acids, amino acids, essential oils, and other undetermined constituents.
I= ethanol-insoluble fraction, including starch, cell wall or structural components such as cellulose, lignin, proteins, pectic substances and hemicellulose (Sinclair, 1984).

after satsuma mandarin fruit were exposed to $^{14}CO_2$ in light. However, previous experiments with grapefruit showed that less than 4% of the ^{14}C-assimilates from fruit photosynthesis were present in juice tissues after comparable periods of time (Chap. III). Albedo of calamondin and satsuma madarin peels is considerably thinner than that of grapefruit. Peel thickness therefore may be important in the extent to which photosynthesis contributes to juice tissues.

Assimilates recovered 7 days after dark $^{14}CO_2$ fixation in juice tissues was partitioned similarly in fruit from arsenated and non-arsenated trees (Table 5-3). In both, a much greater percentage of ^{14}C-assimilates were localized in juice tissues than had been after light $^{14}CO_2$ fixation, although the total amount was far less. A higher ratio of ethanol-soluble to ethanol-insoluble assimilates in juice tissues suggested a different fate for products of dark vs. light CO_2 fixation. The ^{14}C-assimilates in the insoluble fraction of peel extracts probably arose either through photosynthetic refixation of respired $^{14}CO_2$, or synthesis of structural components from amino acids produced after dark $^{14}CO_2$ fixation.

Assimilate partitioning into organic acids of fruits from arsenated and non-arsenated trees was similar after either light or dark CO_2 fixation in fruit but greater amounts of ^{14}C-photosynthates from leaves were recovered in the anion fraction (predominantly organic acids) of fruit from arsenated trees (Table 5-4). Also, the total amounts of labeled and unlabeled photosynthates converted to organic acids of fruit was estimated from total CO_2 fixation, and found to be larger in arsenated than non-arsenated trees. The result might initially appear to conflict with the role of arsenate in

Table 5-4. Assimilate partitioning into the anion fraction (predominantly organic acids) of juice tissues of arsenated and non-arsenated calamondin trees 7 days after a 1 h exposure of either leaves or fruits[z] to $^{14}CO_2$ in light or dark.

Treatments	dpm recovered in anion fraction of juice tissues (dpm 10^3 g^{-1})	Anion fraction expressed as total C fixed (μmol g^{-1})	% of anion fraction recovered in juice tissues (%)
Leaf (Light)			
Arsenated	460.2 ± 50.3	0.296 ± 0.032	33.30 ± 0.54
Non-arsenated	243.0 ± 81.4	0.156 ± 0.052	33.16 ± 2.02
Fruit (Light)			
Arsenated	66.0 ± 13.7	0.039 ± 0.008	32.84 ± 3.92
Non-arsenated	73.5 ± 14.0	0.043 ± 0.008	34.32 ± 1.03
Fruit (Dark)			
Arsenated	3.8 ± 1.1	0.004 ± 0.001	63.77 ± 2.20
Non-arsenated	4.1 ± 0.4	0.004 ± 0.001	60.13 ± 5.99

[z] Fruit were 3-months old.

decreasing the acidity of citrus fruit, but when ^{14}C-phtosynthates in the anion fraction of juice tissues are expressed as a percentage of the amount translocated into these tissues, arsenated and non-arsenated fruit do not differ (Table 5-4). Arsenated trees reportedly show no detectable increases in arsenate of juice tissues (Gilfillan et al., 1976; Rice et al., 1985). Therefore, greater photosynthate export from leaves of arsenated trees could be responsible for results observed here.

Data in table 5-4 also indicate that organic acids in citrus fruit may arise from both light and dark CO_2 fixation by fruit as well as photosynthesis in leaves. The largest contribution to organic acid synthesis was from leaf photosynthesis, followed by fruit CO_2 fixation in light. More than half of the ^{14}C-assimilates resulting from dark CO2 fixation in fruit were partitioned into the anion fraction, but resulted in only a very small total amount of organic acids in calamondin fruit. This differs from previous findings for grapefruit, which showed that dark CO_2 fixation was a major source of organic acids in fruit (Chap. IV). The different fruit size and peel characteristics may affect the amount of respiratory CO_2 retained inside juice tissues, which in turn could influence dark CO_2 fixation and its contribution to organic acids.

Conclusions

The effects of arsenate spray on calamondin fruit acidity were variable, but in June experiments, application of arsenate to whole trees or attached, individual fruit decreased acidity of juice tissues. Arsenical residue in leaves is therefore not necessarily

involved in the typical response to whole-tree sprays. However, effects of arsenate on leaves and/or fruit peel are likely to be important under field conditions because arsenical residues in juice tissues do not differ significantly after arsenate spray (Gilfillan et al., 1976; Rice et al., 1985).

Leaf metabolism appeared to be altered by whole-tree arsenate treatments in the present study, but not that of fruits. Partitioning of ^{14}C-assimilates 7 days after light and dark $^{14}CO_2$ fixation by leaves and fruits of calamondin trees showed that greater percentages of leaf photosynthates were translocated to fruit of arsenated trees. A larger portion of the total assimilates were thus found in the organic acid fraction of fruit. Less total photosynthate was recovered in arsenated trees, however, possibly due to elevated respiratory rates of arsenate-treated leaves reported by Miller et al. (1933). Percentages of ^{14}C-assimilates partitioned into organic acids did not differ significantly if derived from $^{14}CO_2$ fixation by fruit.

The contribution of fruit photosynthesis to growth of calamondin juice tissues appears to be greater than that of grapefruit (23% vs less than 4%) and may be related to differences in peel thickness.

CHAPTER VI
ENZYMES OF ORGANIC ACID SYNTHESIS AND METABOLISM DURING DEVELOPMENT OF NORMAL AND LOW ACID GRAPEFRUIT MUTANTS

Introduction

The organic acid content of fruit is a critical factor in flavor and overall quality. Seasonal and developmental changes in organic acids, as well as associated synthesis and degradation, have been widely studied in many fruit crops (Thimann and Bonner, 1950; Ulrich, 1970; Sinclair, 1984). Several hypotheses have been proposed to account for acid biosynthesis, based particularly on data from grape (Ulrich, 1970; Ruffner et al., 1983) and citrus (Bartholomew and Reed, 1948; Varma and Ramakrishnan, 1956; Huffaker and Wallace, 1959; Buslig, 1971; Akao and Tsukahara, 1980b). Organic acids in grape vines, for example, can be transported from leaves to fruit (Ulrich 1970), although recent evidence indicate this is not likely the main source of fruit acids (reviewed by Ruffner, 1982). In citrus, reciprocal grafts between sour and sweet lemons suggested that the primary mechanism of organic acid synthesis is probably localized in the fruit itself (Erickson, 1957).

Enzymes associated with organic acid accumulation in citrus fruit have been investigated and reviewed (Ericson, 1968; Kefford and Chandler, 1970; Vandercook, 1977; Bruemmer et al, 1977; Sinclair, 1984). Dark CO_2 fixation via phosphoenolpyruvate carboxylase (PEPc) was suggested to be associated with organic acid synthesis in citrus

fruit (Huffaker and Wallace 1959). Activity of PEPc is higher during the period of rapid organic acid accumulation in several citrus fruits (Clark and Wallace, 1963). However, the same and other studies showed that the PEPc acitivities among different citrus cultivars did not correlate with respective fruit acid levels (Clark and Wallace, 1963; Bogin and Wallace, 1966). Phosphoenolpyruvate carboxylase activity was highest in citrus fruit with lowest acidity. The relationship between acid accumulation and the sequence of reactions from CO_2 fixation to citrate synthesis was thus found to be more complicated than initial hypothesis had proposed (Bogin and Wallace, 1966).

Dark $^{14}CO_2$ fixation in lemon fruit, however, resulted in extensive labeling of organic acids (Young and Biale, 1968). Similar results have also been reported for a wide range of citrus fruit (Vu et al., 1985; Chapter IV). Fruit assimilation of CO_2 in the dark was found to be a major source of organic acids in developing grapefruit (Chapter IV). Dark CO_2 fixation also occurs in flavedo and albedo, but does not result in as extensive an accumulation of organic acids (Bean and Todd, 1960).

Citrate synthase and aconitase in the tricarboxylic acid (TCA) cycle are two enzymes directly associated with synthesis and degradation of citric acid. Both enzymes have been reported in leaves and fruit of several citrus varieties (Ramakrishnan and Varma, 1959; Srere and Senkin, 1966; Buslig, 1971; Hirai and Ueno, 1977). Ramakrishnan and Varma (1959) found that in lemon fruit, activity of citrate synthase increased and that of the degradative enzymes, aconitase and isocitrate dehydrogenase, decreased during the period when the acid content rose from 0.9% to 4.0%. Bruemmer et al. (1977)

reviewed the enzyme systems controlling citric acid synthesis in citrus and suggested that citrate synthase was a probable regulatory enzyme for this process as well as for organic acid degradation in citrus fruit. Buslig (1971) suggested that an asynchrony between citrate synthesis and degrading enzymes, such as isocitrate dehydrogenase, might occur during citrate accumulation. Bogin and Wallace (1967) proposed that citramalate, which inhibits aconitase, may contribute to the acid accumulation in citrus fruits. However, there is yet no direct evidence demonstrating that citrate synthase and aconitase are actually involved in acid accumulation by citrus fruit.

Excellent material for comparison of acid metabolism is provided by plants which are genetically identical, or nearly so, except for attributes affecting fruit acidity. Such clones have been developed by bud irradiation of 'Foster' pink grapefruit (Hearn, 1986). Two of the seedless mutations which arose apparently differ from one another in levels of fruit acid; one normal, and the other low.

The first objective of this study was to characterize developmental changes in activities of enzymes potentially associated with acid accumulation in grapefruit tissues. The second was to compare such data from the grapefruit mutants which had either normal or low fruit acidity. Combined results should help clarify mechanisms affecting the acid metabolism in citrus fruit.

Materials and Methods

Plant Material

Grapefruit mutants with normal and low levels of fruit acid (seedless clone # 1-26-39 and 1-26-51, respectively) were derived from 'Foster' pink grapefruit (Citrus paradisi, Macf.) by bud irradiation (Hearn, 1986). All material was propagated vegetatively. Experimental trees were 4-year old on sour orange rootstock in Lake Wales, FL. The two seedless mutants appear to differ from one another in their fruit acidity. Isozyme banding analysis of several enzymes and other data showed no significant differences in characteristics tested between the two clones (Yen, unpublished data, 1985).

Sampling

Fruits were randomly harvested from 1 to 2 m above ground on the canopy exterior on May 7, June 25, July 27, and September 10 (approximately 40-day intervals), after 2, 3.5, 5, and 6.5 months' growth, respectively. One fruit was harvested from each tree and 4 trees were used as 4 replications. Fruit were dissected into flavedo, albedo and juice tissues, frozen immediately with liquid nitrogen, and stored at -80°C until use.

Acid Levels

Acidity was determined by titration. Five g of each tissue were homogenized with a Polytron (Brinkmann Instruments, Westbury, N.Y.) in 50 ml of deionized water. A 5 ml aliquot was again diluted 5:45 (v/v) and titrated to an endpoint of pH 8.2 (using phenolphthalein indicator) with 0.01 N NaOH at 25°C.

Enzyme Assays

Samples for enzyme assays consisted of either 1 g of flavedo (outer, pigmented peel), 1 g of albedo (inner, non-pigmented peel), or 4 g of juice tissues. Each was homogenized at 0 to 4°C using Polytron (Brinkmann Instruments, Westbury, N.Y.) with 10 ml of 0.2 M Tris buffer (pH 8.2), 10 mM isoascorbate, and 0.1% Triton x-100 as described by Hirai and Ueno (1977). The homogenate was filtered through 6 layers of cheese cloth and centrifuged at 1000 x g for 10 min. Supernatant was used to assay protein levels and aconitase activity, while other assays were conducted after removal of sugars, acids, and other low molecular weight compounds (Hirai and Ueno 1977). Extract was passed through a 10 g, Sephadex G-25-150 (Sigma, St Louis, MO.) column (15 X 2.2 cm) previously equilibrated with 0.2 M Tris buffer (pH 8.2) (Hatch and Oliver, 1978). The entire procedure was conducted at 0-4°C.

Protein was quantified according to Bradford (1976), using bovine serum albumin standards.

Enzymes were assayed spectrophotometricaly at about 25°C. Phosphoenolpyruvate carboxylase (EC 4.1.1.31) activity was determined by measuring the decrease in NADH (A_{340nm}) during a coupled reaction with malate dehydrogenase (Lane et al., 1969). The assay medium contained 40 mM Tris buffer (pH 8.5), 10 mM $KHCO_3$, 2 mM $MgCl_2$, 2mM PEP, 0.5 mM glutathione, 0.06 mM NADH, and extract, in a final volume of 2.5 ml. The reaction was initiated by addition of extract. Extracts of grapefruit tissue had high enough activity of malate dehydrogenase (MDH), that addition of exogenous enzyme was not necessary.

Malate dehydrogenase (EC 1.1.1.37) was assayed as described by

Asahi and Nishimura (1973) in a reaction mixture of 75 mM phosphate buffer (pH 7.0), 0.1 mM NADH, 0.75 mM oxaloacetic acid (OAA) and extract in a final volume of 3 ml. The reaction was initiated by addition of extract and quantified by spectrophotometric measurement of decreases in NADH.

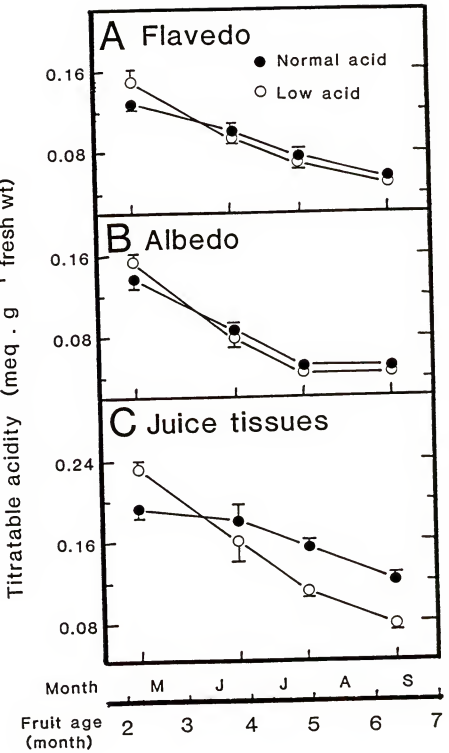
Citrate synthase (EC 4.1.3.7) activity was determined according to Srere (1969), by following production of mercaptide (A_{412nm}), formed during the reaction of CoASH with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The assay medium contained 40 mM Tris buffer (pH 9.0), 40 µM DTNB, 80 µM Acetyl CoA, 80 µM oxaloacetic acid, and extract in a final volume of 2.5 ml.

Aconitase (EC 4.2.1.3) was assayed by measuring the disappearance of cis-aconitate absorption at 240 nm (Hirai and Ueno, 1977). The reaction medium contained 40 mM Tris buffer (pH 7.5), 100 mM NaCl, 0.1 mM cis-aconitate, and tissue extract in a final volume of 2.5 ml. The reaction was initiated by addition of extract.

Results

Acidity decreased gradually during development of all fruit tissues from both the normal- and low-acid grapefruit clones (Fig. 6-1). This was due primarily to fruit expansion, because total acid per fruit increased (data not shown). General changes in titratable acidity of juice tissues were similar to those of other grapefruit (Ting and Vines, 1966). Juice tissue acidity in the present study was consistently greater than that of flavedo or albedo. Acid levels were also significantly greater in all tissues of very young fruit from the low-acid clone when compared to those of its counterpart known to have

Fig. 6-1. Acid levels during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.


A Flavedo
Normal acid
Low acid
0.16
0.08
B Albedo
0.16
0.08
C Juice tissues
0.24
0.16
0.08
Titratable acidity (meq . g⁻¹ fresh wt)
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

normal acidity at harvest. However, mean titratible acidity of juice tissues from the low-acid clone dropped rapidly between the 2nd and 4th months of fruit growth (May and June), so that by the 5th month (July), acid levels were significantly less than those of fruit from clones with normal acid levels.

Protein content decreased in flavedo, albedo, and juice tissues throughout fruit development (Fig. 6-2). Differences were seldom significant between grapefruit with normal acid levels when compared to those of its low acid counterpart. Protein content was maximal in the youngest fruit sampled (May, 2 mo) and was greatest in juice tissues and flavedo. Levels in all tissues dropped during subsequent fruit development, but did so most rapidly in expanding juice tissues.

Decreased protein concentration has also been reported during enlargement (stage II) of 'Valencia' orange fruit, but was apparently accompanied by an increase in soluble nitrogen (Bain, 1958). Ramakrishnan (1971) suggested that the amino acids derived from protein breakdown during this stage could be utilized as respiratory substrates. Ribulose biphosphate carboxylase is not the primary protein in citrus peel (Goldschmidt, 1986), but may acount for the higher protein content observed in flavedo. Degradation of RuBP carboxylase subunits does occur during final maturation of citrus peel (Goldschmidt, 1986).

All enzyme activities (Fig 6-3 to 6-6) are expressed per g fresh weight so that they can be more readily compared to organic acid accumulations per g fresh weight.

Greatest activities of PEPc per g fresh weight were recorded for extracts from the youngest juice tissues, which were also at their

Fig. 6-2. Protein content during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.

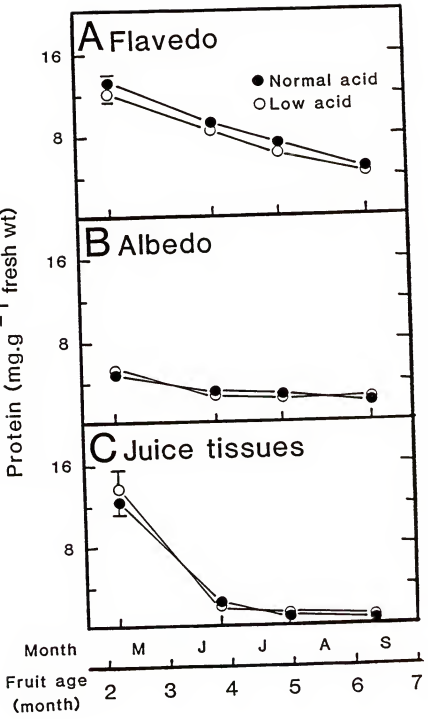
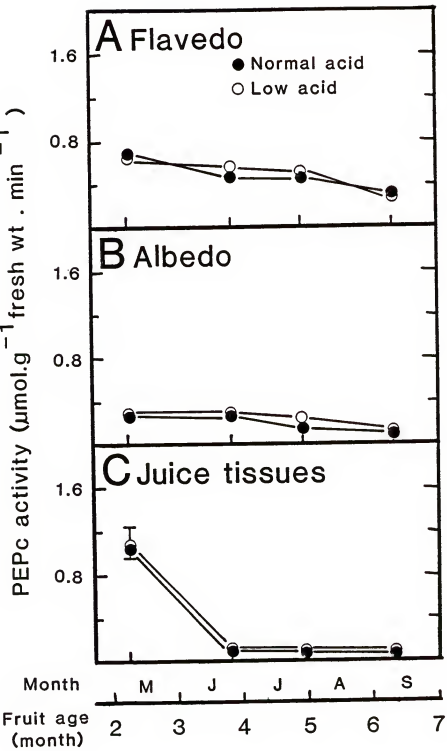

A Flavedo
Normal acid
Low acid
16
8
B Albedo
16
8
C Juice tissues
16
8
Protein (mg.g $^{-1}$ fresh wt)
Month
M
J
J
A
S
Fruit age
(month)
2
3
4
5
6
7

Fig. 6-3. Phosphoenolpyruvate carboxylase (PEPc) activity during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.


A Flavedo
Normal acid
Low acid
B Albedo
C Juice tissues
1.6
0.8
PEPc activity (µmol.g^{-1}fresh wt . min^{-1})
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

most acid stage of development (Fig. 6-3). Activity dropped rapidly during subsequent fruit growth, however, to levels less than those from other tissues of the same fruit. Phosphoenolpyruvate carboxylase from flavedo and albedo was generally more active early in development and decreased gradually. Differences in PEPc activities were not significant between extracts of fruit from clones with normal vs. low acid levels. The amount and trend of PEPc activity during fruit development was similar to that reported for soybean fruits and pea pods, which reportedly reassimilate substantial respiratory CO_2 (Quebedeaux and Chollett, 1975; Atkins et al., 1977). However, the activity of PEPc from individual tissues was apparently lower than those of flowers and leaves of 'Valencia' orange (Vu et al., 1985).

Overall, trends in MDH activities per gram fresh weight were very similar to those observed for PEPc (Fig. 6-4). Again, differences were not significant between activities in extracts from fruit of clones with normal vs. low acid levels. Developmental changes in activities of citrate synthase in extracts of fruit tissues differed from those of PEPc and MDH, primarily in the extent to which activity of flavedo extracts was elevated between the 2nd and 6th months of growth (Fig. 6-5). In albedo, citrate synthase activities per g fresh weight changed less during development, although, there was a slight decrease from July to September. In contrast, citrate synthase in juice tissues was similar to PEPc and MDH in that it was most active in extracts of young fruit , when the acidity was greatest. Activities of citrate synthase from grapefruit tissues were comparable to those previously reported for lemons and oranges (Srere, 1966) and not significantly different for normal and low acid grapefruit.

Fig. 6-4. Malate dehydrogenase (MDH) activity during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.

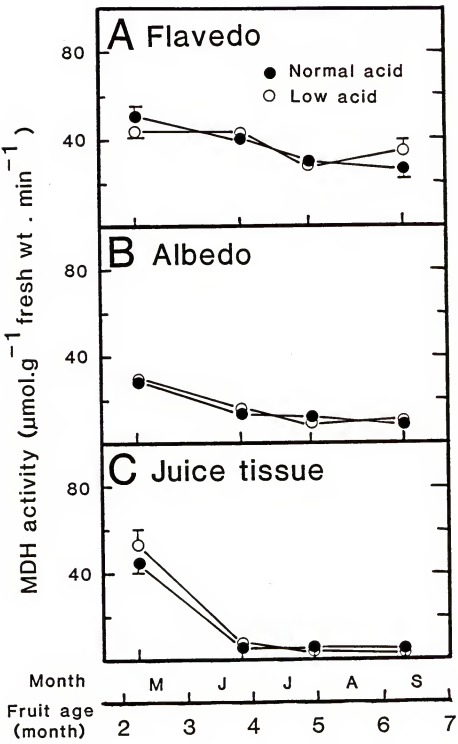

A Flavedo
Normal acid
Low acid
B Albedo
C Juice tissue
80
40
MDH activity (μmol.g⁻¹ fresh wt . min⁻¹)
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

Fig. 6-5. Citrate synthase (CS) activity during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.

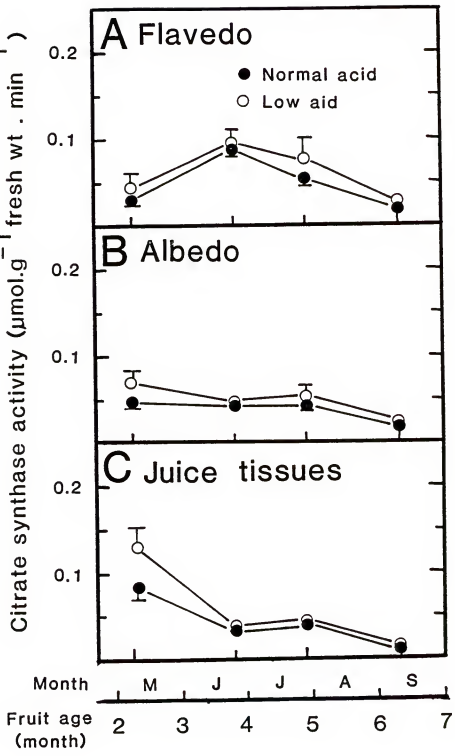
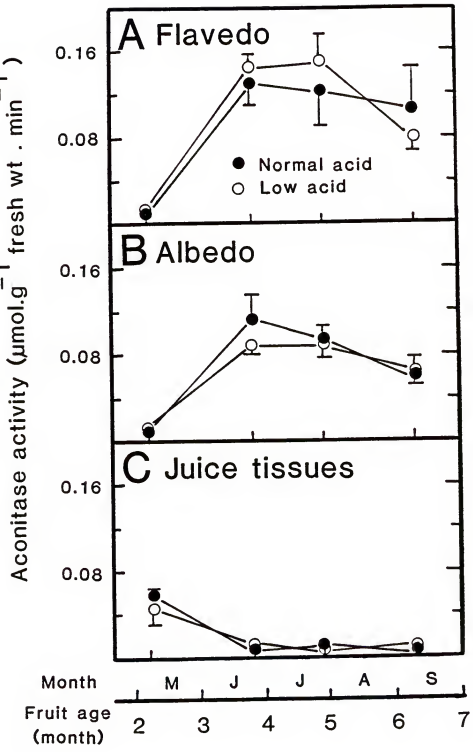

A Flavedo
Normal acid
Low aid
B Albedo
C Juice tissues
0.2
0.1
Citrate synthase activity (μmol.g⁻¹ fresh wt . min⁻¹)
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

Fig. 6-6. Aconitase (AC) activity during development of flavedo (outer, pigmented peel) (A), albedo (inner, non-pigmented peel) (B), and juice tissues (C) of fruit from grapefruit with normal acid levels ('Foster' pink, seedless clone # 1-26-39), or low acid levels ('Foster' pink, seedless clone # 1-26-51). Vertical bars denote the SE of 4 replications and are not pictured where smaller than symbols used.


A Flavedo
B Albedo
C Juice tissues
0.16
0.08
Normal acid
Low acid
Aconitase activity (µmol.g^{-1} fresh wt . min^{-1})
Month
M
J
J
A
S
Fruit age (month)
2
3
4
5
6
7

Aconitase activity in flavedo and albedo increased dramatically during the 3rd month of fruit growth (from May to June) and remained near this level throughout the rest of development (Fig. 6-6). In the juice tissues, aconitase activity changed in a manner similar to that of PEPc, MDH, and citrate synthase. Activity was highest in extracts of the young fruit (May), when acid concentration was greatest. Aconitase activity from young fruit was less than that of citrate synthase, and did decrease more rapidly, possibly contributing to acid accumulation in juice tissues during these periods.

Ratios of citrate synthase to aconitase activity were greatest from June to July when rates of acid accumulation were most rapid in both grapefruit clones (Table 6-1).

Discussion

The identification of PEPc activity in grapefruit was not surprising, due to its presence in a wide variety of tissues, and its many-faceted function in C_3 plants (reviewed by Latzko and Kelly, 1983). Phosphoenolpyruvate carboxylase activity has also been reported in leaves, roots, and fruit of rough lemon, trifoliate orange, and 'Valencia' orange (Huffaker and Wallace, 1959), and flower buds of 'Valencia' orange (Vu et al., 1985).

Of greatest interest, however, is the proposal by Huffaker and Wallace (1959), that dark CO_2 fixation via PEPc may be the source of organic acids in citrus fruit (Huffaker and Wallace, 1959). Previous results have indicated that dark CO_2 fixation is a major source of organic acids in citrus fruit (Chapter IV) and that the extent of its contribution to total fruit acidity becomes more important during

Table. 6-1. The rate of organic acid accumulation and ratio of citrate synthase to aconitase in extracts from juice tissues of normal- and low-acid grapefruit during development.[z]

Grapefruit clones	Rate of organic acid accumulation in juice tissues		
	May to June	June to July	July to Sept
	(meq day^{-1} juice $tissue^{-1}$)		
Normal acidity[y]	0.25 ± 0.01	0.34 ± 0.04	0.20 ± 0.06
Low acidity	0.19 ± 0.02	0.19 ± 0.02	0.07 ± 0.07

	Ratio of citrate synthase to aconitase activity			
	May	June	July	Sept
	(citrate synthase/aconitase)			
Normal acidity	1.41	7.02	3.88	2.51
Low acidity	2.92	4.71	5.29	2.07

z Fruit were harvested in May, June, July, and September after approximately to 2, 3.5, 5, and 6.5 months' growth, respectively.

y The normal and low acid grapefruit clones are seedless mutants of 'Foster' pink.

development. However, data in the present study show that although PEPc is quite active in extracts from juice tissues of very young fruit, activity drops rapidly during development (Fig. 6-3), despite continued accumulation of organic acids in tissues. Further, PEPc activities of flavedo and albedo extracts (relatively low acid tissues) were higher than those from juice tissues (higher acidity) at most developmental stages (compare Fig. 6-3 and 6-4). The total activity of PEPc per fruit either in flavedo or in albedo was also greater than that in juice tissues (data not shown). Phosphoenolpyruvate carboxylase activity has previously been found not to coincide with the acid concentration in the tissues of origin (Clark and Wallace, 1963; Akao and Tsukahara, 1979). Phosphoenolpyruvate carboxylase may therefore be an important source of organic acids in citrus fruit (Chapter IV), but is not necessarily the mechanism controlling accumulation.

A possible function of PEPc in citrus fruit may also be refixation of respiratory CO_2. Recapture of respiratory CO_2 by PEPc has been reported in soybean, pea pod, and tomato (Quebedeaux and Chollet, 1975; Atkins et al., 1977; Laval-Martin et al., 1977). Comparison of CO_2 exchange from intact grapefruit in light and dark indicated that large amounts of refixation occurred in the light (data not shown). Although the PEPc activity of flavedo was higher than that of juice tissues at later stages of development, refixation of CO_2 in flavedo probably occurred via ribulose-biphosphate carboxylase. Phosphoenolpyruvate carboxylase may predominate for CO2 refixation in the fruit interior. Still, PEPc also acts as a cellular pH-stat and has maximum activity near pH 8 (Davies, 1973). A CO_2 concentration of

5% can reportedly lower intracellular pH and affect the enzyme activity (Bown, 1985). Internal CO_2 levels of more than 10% were reported in navel orange fruit 2 days after storage at 20°C (Eaks and Ludi, 1960). The involvement of PEPc in refixation of respiratory CO_2 by juice tissues may therefore be complicated by higher internal CO_2 concentrations in citrus fruit.

Formation of fruit acids via PEPc may be associated with the replenishment of TCA cycle intermediates as suggested by Latzko and Kelly (1983). This hypothesis also includes a close relationship between PEPc and MDH activities (Latzko and Jelly, 1983). In vivo metabolism was not examined in the present study, but assays of maximal activity for both enzymes showed extensive similarity between changes in overall capacity of PEPc and MDH (Fig. 6-3 and 6-4). As noted for PEPc, however, combined activities of PEPc and MDH do not correspond to acid accumulation within different tissues or to change in acidity which occur during development.

Citrate synthase has been reported in both mitochondria and glyoxysomes of maize scutellum (Barbareschi et al., 1974). In citrus fruit, however, Vines (1968) found that citrate synthase was active in mitochondrial fractions, but not detectable in the cytoplasm (cited from unpublished data in Ting and Attaway, 1971). The existence of the glyoxylate cycle has been demonstrated in young satsuma mandarin fruit by feeding sodium glyoxylate-1-^{14}C to juice vesicles, but, the rate was relatively low when compared with that of the TCA cycle (Kobota and Akao, 1973b). Activities of citrate synthase measured in extracts from developing citrus fruit (Fig. 6-5) are probably those of the mitochondrial enzyme.

Data on citrate synthase and aconitase in the present paper provide evidence in support of the following hypothesis. Several authors have discussed the possibility that combined action of citrate synthase and the two subsequent enzymes in the TCA cycle, aconitase and isocitrate dehydrogenase might control the synthesis and degradation of citrate in citrus fruit (Ramakrishnan and Varma, 1959; Buslig, 1971; Bruemmer et al., 1977). Here, results in Fig. 6-5 and 6-6 show, first, that extracts of fruit tissues with high acid levels had greater activities of citrate synthase relative to aconitase. This was evident at all stages of development, but was most pronounced during periods of most extensive acid accumulation (Table 6-1). In contrast, extracts of tissues which had lower acid levels, flavedo and albedo, also had less active citrate synthase relative to aconitase at all but the earliest stage of development examined.

A second line of evidence compared activities of citrate synthase and aconitase between young fruit of grapefruit clones with normal and low acid levels. Early in development, low acid fruit was more acid than that of their otherwise higher acid counterparts. At this time, citrate synthase activity was significantly higher in extracts of fruit from the low acid clone, and aconitase activity was lower. Activities of the two enzymes thus coincided well with initial acid levels in fruit from both grapefruit clones. Significant differences in these enzyme activities were not evident between fruit of the two clones at later stages of growth, but small differences may have contributed to different long term acid accumulations.

A relationship between overall efficiency of mitochondrial metabolism and the acid levels in citrus fruit has been reported in

other studies (Bogin and Wallace, 1967; Vines and Metcalf, 1967). Sweet lemons were found to have more active oxidation and phosphorylation than sour lemons (Bogin and Wallace, 1967). Citrus varieties with less fruit acid also showed a higher efficiency in utilization of respiratory substrates (Buslig, 1971). At less acid stages, developing citrus fruit more effectively oxidized intermediates of the TCA cycle (reviewed by Vandercook, 1977). A partial block in the TCA cycle was presumed to have occurred to a greater extent in sour than sweet fruit. Studies of satsuma mandarin juice vesicles indicated that synthesis of citrate and malate was favored when the TCA cycle was blocked by various inhibitors at different locations (Kubota and Akao, 1973). Preferential synthesis of malate or citrate depended on where the TCA cycle was blocked. Therefore, aconitase may play an important role in sour fruit which accumulate the higher levels of citrate.

Factors other than the citrate synthase-aconitase enzyme system cannot be excluded. Enzymes of the TCA cycle are known to be controlled by many factors such as energy charge, pH, and compartmentalization of substrates (Wiskich, 1980). The supply of precursors to TCA intermediates including the amount of carbohydrates transported from leaves might also be involved as indicated in citrus by an association between altered glycolysis and shifts in patterns of acid accumulation (Parekh and Shah, 1971; Parekh et al, 1970; Ruffner and Hawker, 1977).

Conclusions

Developmental examination and comparison of enzyme activities in extracts of flavedo, albedo, and juice vesicles indicated that dark CO_2 fixation via the combined reactions of PEPc and MDH is an important source of organic acid synthesis, but overall control of organic acid accumulation apparently exists at steps other than in the PEPc-MDH pathway.

The greater activity of citrate synthase and lesser activity of aconitase in tissues with higher acid levels indicated that the balance between of these two enzymes may influence accumulation by affecting synthesis and degradation of citrate in citrus fruit.

CHAPTER VII
CONCLUSIONS

Distribution of labeled assimilates after light and dark $^{14}CO_2$ fixation by leaves and fruit was as follows:

1. Export products of both light and dark $^{14}CO_2$ fixation in leaves were deposited primarily in juice sacs of fruit. More of the assimilates were retained in leaves after dark CO_2 fixation.
2. Fruit photosynthesis gave rise to assimilates which remained primarily in flavedo and albedo 24 h later, thus apparently contributing little to growth of juice tissues.
3. ^{14}C-assimilates from dark $^{14}CO_2$ fixation in intact fruit were localized primarily in juice tissues.

An assessment of the relative C contributed by each assimilate source to fruit development at various stages of growth indicated that leaf photosynthesis was most important when fruit were very young. As fruit expanded, the proportion of C contributed by fruit CO_2 fixation either in light or in the dark increased.

Sources of organic acids were the same as those of total C only in very young fruit. Leaves were the primary source of assimilates for organic acid formation early in fruit development. However, dark CO_2 fixation by fruit became a major, and perhaps the predominant source of organic acids later in development, despite its relatively minor contribution to total C. About 90% of anion fraction of ^{14}C-assimilates recovered from dark $^{14}CO_2$ fixation in juice tissues of

5-month-old fruit was citrate and malate. The ratio of labeled citrate to malate was far less than that of total levels in juice tissues possibly due to either slow turnover of malate in vacuoles or continuous refixation in the dark.

Acid concentration decreased and total acidity increased continously throughout development of fruit from both normal- and low-acid grapefruit clones, but values were significantly less in low-acid grapefruit after 5 or more months' growth. In more mature fruit, the citrate to malate ratio was also significantly less in fruit from the low- vs. high-acid clone, 9:1 and 14:1, respectively. Significant differences were not evident in amounts of ^{14}C-assimilates partitioned into organic acids in fruit of normal- and low-acid grapefruit clones, but may have resulted from the relatively short initial exposure time (1 h) compared to the duration of fruit development.

Developmental examination and comparison of enzyme activities in extracts of flavedo, albedo, and juice vesicles indicated that dark CO_2 fixation via the combined reactions of PEPc and MDH is an important source of organic acid synthesis, but overall control of organic acid accumulation apparently exists at steps other than in the PEPc-MDH pathway.

The greater activity of citrate synthase and lesser activity of aconitase in tissues with higher acid levels indicated that the activities of these two enzymes together may be important to control of citrate synthesis and degradation of citrate in citrus fruit.

June application of arsenate to calamondin trees or attached, individual fruit decreased acidity of juice tissues, indicating that

arsenical residue in leaves is not necessarily involved in the typical response to whole-tree sprays. However, effects of arsenate on leaves and/or fruit peel are likely to be important under field conditions because arsenical residues in juice tissues do not differ significantly after arsenate spray (Gilfillan et al., 1976; Rice et al., 1985).

Leaf metabolism appeared to be altered by whole-tree arsenate treatments in the present study, but not that of fruits. Partitioning of ^{14}C-assimilates seven days after light and dark $^{14}CO_2$ fixation by leaves and fruits of calamondin trees showed that a greater percentage of leaf photosynthates was translocated to fruit of arsenated trees. A larger portion of the total assimilates were thus found in the organic acid fraction of fruit. Less total photosynthates were recovered in arsenated trees, however, possibly due to elevated respiratory rates of arsenate-treated leaves reported by Miller et al. (1933). Percentages of ^{14}C-assimilates partitioned into organic acids did not differ significantly if derived from $^{14}CO_2$ fixation by fruit.

The contribution of fruit photosynthesis to growth of calamondin juice tissues appears to be greater than that of grapefruit (23% vs less than 4%) and may be related to differences in peel thickness.

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BIOGRAPHICAL SKETCH

Chung-Ruey Yen was born in Taipei, Taiwan, The Republic of China, on Jan. 5, 1953. He entered the Department of Horticulture, National Chung-Hsing University in Taichung, Taiwan, in 1971. He received his Bachelor of Science degree in 1975 and entered the Department of Horticulture, National Taiwan University, Taipei, for a graduate program. He studied on postharvest physioloy of 'Bartlett' pear under the direction of Profs. Ming-nang Chiang and Pin-li Tsai. He received his Master of Science degree in 1977 and spent another two years as second lietenant in the army where he was in charge of affairs about horticulture and administration. After he was discharged from the army, he got his first horticultural job as a technician and was responsible for field experiments at the Agricultural Engineering Research Center in 1980. In order to obtain international experience in agricultural research and production, he shifted to the Asian Vegetable Research and Development Center (AVRDC), Tainan, Taiwan, in 1981. He was a research assistant and worked for Dr. Shanmugasundaram on soybean breeding for 2 years at AVRDC and put much emphasis on development and breeding of vegetable soybean, a distinctive oriental protein-rich vegetable. After that, he was an assistant horticulturist working on tropical and subtropical fruits at Chia-Yi Agricultural Experiment Station, a branch of Taiwan Agricultural Research Institute. He was responsible for the breeding of litchi and passionfruit and management of several tropical fruits such as mango,

guava, macademia nut, and longan. He entered the Ph.D. program at the University of Florida in the spring, 1985, under the guidance of Dr. Karen E. Koch. During this period, he was sponsored a scholarship for two and half years for Ph.D. study from the Council of Agriculture, Republic of China, and a graduate assistantship by Fruit Crops Department. He will receive his degree in December, 1987.

Chung-Ruey Yen was married in 1982 to Li-Lin Chou. Upon completion of his graduate studies he and his wife will return their country.

Chung-ruey Yen is a life member of Chinese Society for Horticultural Science. Currently, he is also a member of Intrenational Society for Horticultural Science, American society for Horticultural Science, American Society for Plant Physiologist, Florida State Horticultural Society and California Rare Fruit Growers.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Karen E. Koch, Chairman
Associate Professor of
Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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